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Studies of vitamin E metabolism, particularly in subjects undergoing exercise and patients with peroxisomal and mitochondrial disorders

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A thesis submitted for the degree of Doctor of Philosophy in the University of London

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In the name of Allah, the most Compassionate, the most Merciful

Abstract

Vitamin E (α-tocopherol) is the major lipid soluble secondary antioxidant in vivo and is important for maintaining the integrity of cell membranes. It may also have more specific functions in vivo including roles in cell signaling and gene expression. Relatively little is known, however, about the details and dynamics of vitamin E metabolism, which may be important in fully understanding its role(s) in vivo. Vitamin E metabolites detected in urine include α-tocopheronolactone (α-TL), with an oxidized chroman ring, and compounds with successively shortened phytyl side chains, including the carboxyethyl-hydroxychromans (CEHC) and carboxymethylbutyl-hydroxychromans (CMBHC). As α-TL can be produced from α-tocopherol by oxidation it may be a potential biomarker of in vivo oxidative stress. The CEHCs are the major urinary metabolites but the cellular localisation of the side-chain shortening process (mitochondria and/or peroxisomes) remains undefined. The aims of my studies were to determine whether α-TL could be a useful biomarker of oxidative stress and define the cellular localisation of the side-chain shortening process of the tocopherols.

Initially methods were established and validated for the measurement of urinary vitamin E metabolites and the F_2-isoprostane, 8isoPGF$_{2\alpha}$ (a non-enzymatically derived isomers of the prostaglandins, formed in vivo by free radical mediated oxidation of arachidonic acid and an accepted biomarker of oxidative stress) by gas chromatography-mass spectrometry (GC-MS).

To investigate whether α-TL was a potential biomarker of in vivo oxidative stress, urinary α-TL and 8-isoprostane were measured in healthy trained and untrained males before and after a standardised endurance exercise regimen. A significant correlation ($p<0.0001$) was found between the concentrations of α-TL and 8-isoPGF$_{2\alpha}$. There was, however, no evidence of an increase in oxidative stress following exercise in the subjects studied.

Studies to investigate the role of peroxisomes and mitochondria in vitamin E metabolism utilized two approaches. The first involved the analysis of urinary vitamin E metabolites of patients with peroxisomal (n=5) and mitochondrial (n=3) disorders, and those suspected of peroxisomal or mitochondrial disorders (n=5) compared to age matched controls (n=19). No consistent and significant differences in metabolite profiles were found between the groups. The second approach involved tissue culture studies to investigate peroxisomal and mitochondrial function. These studies suggested that both organelles played a role in tocopherol metabolism.

To conclude, firstly α-TL was not shown conclusively, by this thesis to be a biomarker of oxidative stress, but its correlation with the independent biomarker, 8-isoPGF$_{2\alpha}$, may suggests that there is still potential in it being a biomarker in other states of oxidative stress such as in sepsis or disease. Therefore the techniques developed and used here may be used in further investigations to answer the question whether α-TL is a biomarker of oxidative. Secondly, the localization of γ-tocopherol metabolism has been shown to involve the

peroxisome and mitochondria, in human liver HepG2 cells, with effects at different stages of metabolism showing similarity to the branched chain fatty acid metabolism pathway.

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Presentations

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TITLE: The possible use of urinary α-tocopheronolactone as a biomarker of oxidative stress

12th FSV (Fat soluble vitamins) group meeting. Rieti, Italy.2003

TITLE: Urinary Vitamin E metabolite, α-tocopheronolactone – a biomarker of in vivo oxidative stress?

Institute of Child Health, London. Open day. 2002

Title as above

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List of abbreviations

ACO	- acyl-CoA oxidase
ALDP	-adrenoleukodystrophy protein
ATP	-adenosine triphosphate
AVED	-ataxia with vitamin E deficiency
AMACR	-α-methylacyl-CoA racemase
BCA	-bicinchoninic acid
BCOX	-branched chain acyl-CoA oxidase
BP	-bifunctional protein
BSTFA	-N,O-bis(trimethylsilyl)trifluoroacetamide
CACT	-carnitine:acyl-carnitine translocase
CEHC	-carboxyethyl hydroxychroman
CMBHC	-carboxymethyl-hydroxychroman
CMHHC	-carboxymethylhexanyl-hydroxychroman
COT	-carnitine octanoyl transferase
CPT-I/II	-carnitine palmitoyl transferase I/II
CRALBP	-cellular retinaldehyde binding protein
DAPI	-4,6-diamidino-2-phenylindole
DHCA	-dihydroxycholestanoic acid
DMSO	-dimethyl sulphoxide
ECD	-electrochemical detection
EIA	-enzyme immuno-assay
ER	-endoplasmic reticulum
ESR	-electron spin resonance
ETF	-electron transfer flavoprotein
FA	-fatty acid
FAD	-flavin adenine dinucleotide
FALDH	-fatty aldehyde dehydrogenase
FBS	-foetal bovine serum
GC-MS	-gas chromatography-mass spectrometry
GSH	-glutathione

Hb	-haemoglobin
HDL	-high density lipoprotein
HPL	-2-hydroxyphytanoyl-lyase
HPLC	-high performance liquid chromatography
HCl	-hydrochloric acid
IRD	-infantile Refsums disease
LCAD	-long chain acyl-CoA dehydrogenase
LCAS	-long chain acyl-CoA synthase
LCFA	-long chain fatty acid
LCHAD	-long chain 3-hydroxyacyl-CoA dehydrogenase
LDL	-low density lipoprotein
LL	-lipoprotein lipase
LOOH	-lipid peroxide
Mb	- myoglobin
MCAD	-medium chain acyl-CoA dehydrogenase
MeOH	-methanol
MDA	-malondialdehyde
MFP	-multifunctional protein
MS/MS	-tandem mass spectrometry
MTBE	-methyl tertiary-butyl ether
MTP	-mitochondrial trifunctional protein
NADPH	-nicotinamide adenine dinucleotide phosphate
NALD	-neonatal adrenoleukodystrophy
PBD	-peroxisomal biogenesis disorder
PBS	-phosphate buffered saline
PFOA	-perfluorooctanoic acid
PFOS	-perfluorooctane sulphate
PhyH	-phytanoyl-CoA hydroxylase
PKC	-protein kinase C
PP_2A	-protein phosphatase type 2A
PPAR	-peroxisome proliferator associated receptor
PUFA	-polyunsaturated fatty acids
PXR	-pregnane X receptor
QC	-quality control

RCDP	-rhizomelic chondrodysplasia punctata
REDOX	-reduction/oxidation
RNS	-reactive nitrogen species
ROS	-reactive oxygen species
RPE	-rating of perceived exertion
SCAD	-short chain acyl-CoA dehydrogenase
SCOX	-straight chain acyl-CoA oxidase
SCPx	-sterol carrier protein X
SIM	-selected ion monitoring
SOD	-superoxide dismutase
SPE	-solid phase extraction
SPF	-supernatant protein factor
α-TA	-α-tocopheronic acid
TAP	-tocopherol associated protein
TBP	-tocopherol binding protein
TCA	-tricarboxylic acid
TFP	-trifunctional protein
TH	-tocopherol
THCA	-trihydroxy cholestanoic acid
α-TL	-α-tocopheronolactone
TLC	-thin layer chromatography
α-TQ	-α-tocopherylquinone
α-TQH_2	-α-tocopheryl hydroquinone
TRP	-tocopherol regulatory protein
α-TTP	-α-tocopherol transfer protein
UCP	-uncoupling protein
UV	-ultraviolet
VLCFA	-very long chain fatty acid
VLDL	-very low density lipoprotein
XALD	-X-linked adrenoleukodystrophy
XDH	-xanthine dehydrogenase
XOD	-xanthine oxidase
ZS	-Zellweger syndrome

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Chapter 1

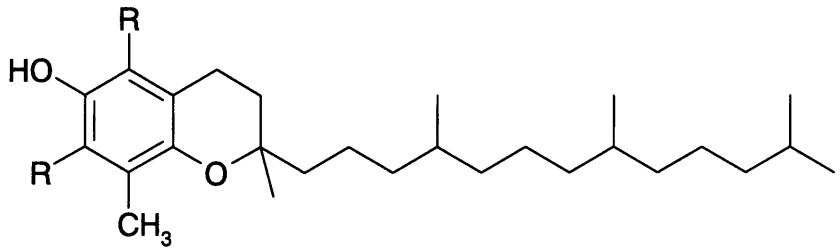
General Introduction

1.1 The History of vitamin E

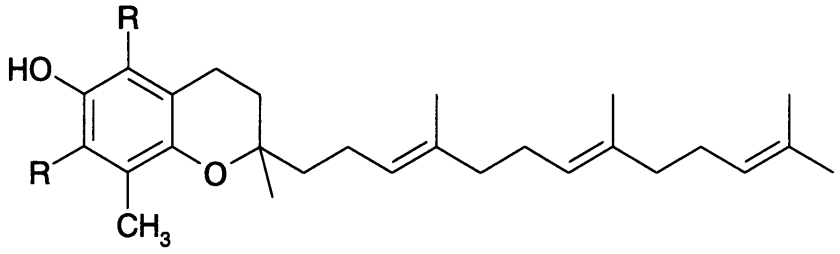
In 1922 Evans and Bishop found that pregnant female rats fed with what they thought was a complete diet, did not give birth to offspring as a result of foetal resorption. However, upon the addition of lettuce to the diet, the foetal resorption defect was corrected and the female rats produced healthy offspring (Evans and Bishop, 1922). It was, therefore, hypothesised that the initial diet had a nutrient deficiency that could be corrected by supplementing with lettuce leaves. In 1936, Evans named this nutrient 'tocopherol', which is a Greek word, meaning 'to bear offspring'. The name tocopherol is now used to refer to a sub-group of vitamin E compounds.

1.2 Structure and nomenclature of vitamin E

All vitamin E compounds consist of a characteristic chromanol ring and carbon side-chain. They comprise two sub-groups; the tocopherols which have a saturated side-chain, and the tocotrienols which have an unsaturarted side-chain with three double bonds (figure 1.1). Both the tocopherols and tocotrienols have four different forms. These forms vary depending on the number and arrangement of the methyl groups on the benzene ring, and are designated, α, β, δ and γ, as shown in figure 1.2.



tocopherols



tocotrienols

Figure 1.1 Chemical structure of the tocopherols and tocotrienols

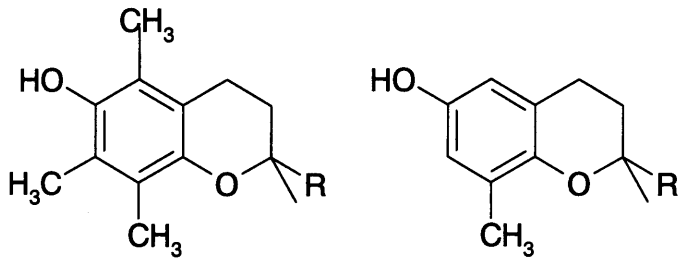


Figure 1.2 The arrangement of methyl groups on the benzene ring of the four different forms of tocopherols and tocotrienols

The structural formula of α-tocopherol (α-TH) was first published in 1938 by Fernholz. The tocopherols, due to their saturated phytyl-chain, have three chiral centres, at carbon positions 2, 4' and 8' (figure 1.3). Rotation depends on the order of the three different groups about these carbon centres and their relative masses to one another, and can occur either as R-rotation or as S-rotation. Naturally occurring vitamin E, exhibits R-rotation at all three centres (*RRR*-tocopherol), whereas synthetic *all-rac*-tocopherol consists of an equimolar racemic mixture of all 8 possible stereo-isomers (*RRR*, *RRS*, *RSR*, *SRR*, *RSS*, *SSR*, *SRS* and *SSS*- tocopherols) (figure 1.3).

1.3 Sources of vitamin E

In nature vitamin E is synthesised only by plants and other photosynthetic organisms (Sattler et al., 2003). The richest dietary source of vitamin E is edible vegetable oils with varying proportions of α, β, δ and γ-tocopherols (TH) and tocotrienols. Generally, *RRR*–α-TH is high in wheat germ, safflower and sunflower oils, *RRR*-γ-TH and tocotrienols are high in soyabean and corn oils, and α/γ-tocotrienols are abundant in palm oil (Traber and Sies, 1996). The THs are present in oil, seed, leaves and other green parts of higher plants. α-TH is mainly present in photosynthetic tissues, while β, δ and γ-TH frequently predominate in non-photosynthetic tissues (Kamal-Eldin and Appelqvist, 1996; Hofius and Sonnewald, 2003).

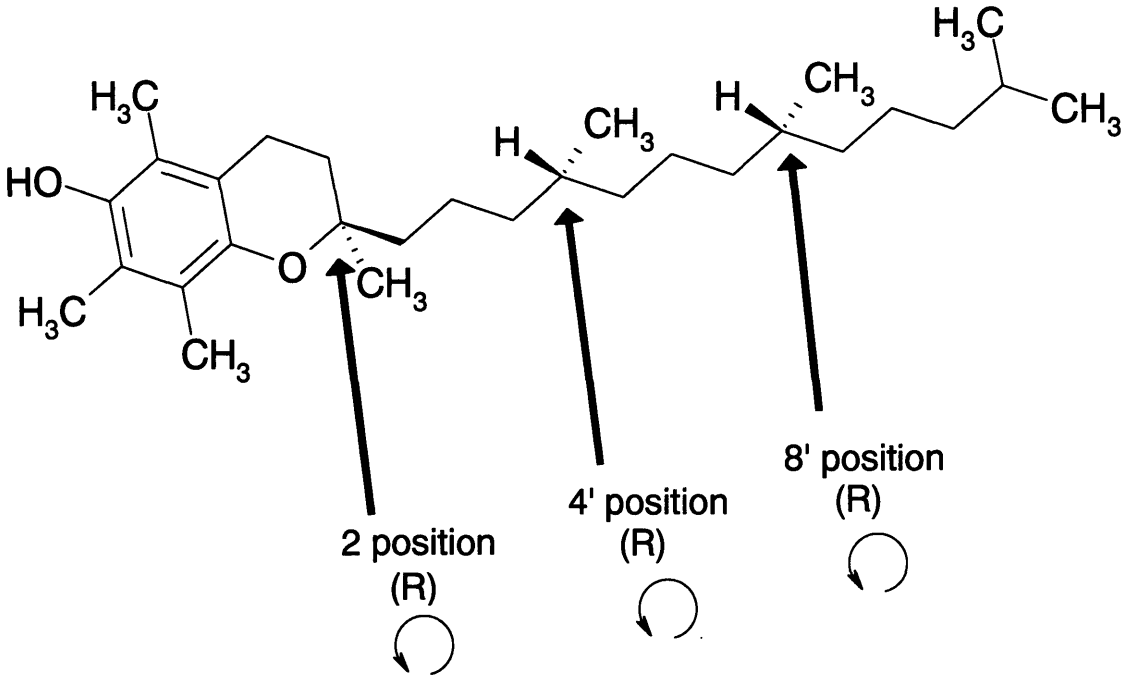


Figure 1.3 The rotation of bonds around the three chiral centres of tocopherol.
[Synthetic vitamin E : *RRR, SRR, SSR,SSS, RSR, RSS,*
RRS, SRS (all possible forms), Natural vitamin E : *RRR*]

The tocotrienols are not found in the green parts of plants but rather in the bran and germ fractions of certain seeds and cereals.

The principal forms of vitamin E in human and mammalian diets vary around the world and depend mainly on the type of plant oils used. In a typical North American diet, due to a widespread use of corn and soya oil, *RRR*-γ-TH is the most abundant and approximately two to four times in excess of *RRR*-α-TH (Vatassery et al., 1983; Behrens and Madere, 1986). Less γ-TH is consumed in European and in South East Asian diets, and tocotrienols are in much higher abundance due to the use of palm oil. Despite these dietary differences generally 90% of vitamin E in human plasma and tissues is in the RRR-α-TH form (Traber et al., 1993).

1.4 Vitamin E absorption and transport

Vitamin E compounds are lipid soluble and as a result their absorption follows a similar process to that of dietary lipids such as triglycerides and cholesterol. The absorption process of dietary lipids comprises 1) emulsification 2) solubilisation within mixed bile salt micelles, 3) uptake by small intestinal cells (enterocytes), 4) packaging within lipoprotein particles (chylomicrons), and 5) secretion into the circulation via the lymphatic system (figure 1.4). Emulsification begins in the stomach to form chyme and is achieved by predominantly mechanical forces. In the small intestine, chyme mixes with pancreatic and biliary secretions. Pancreatic lipase

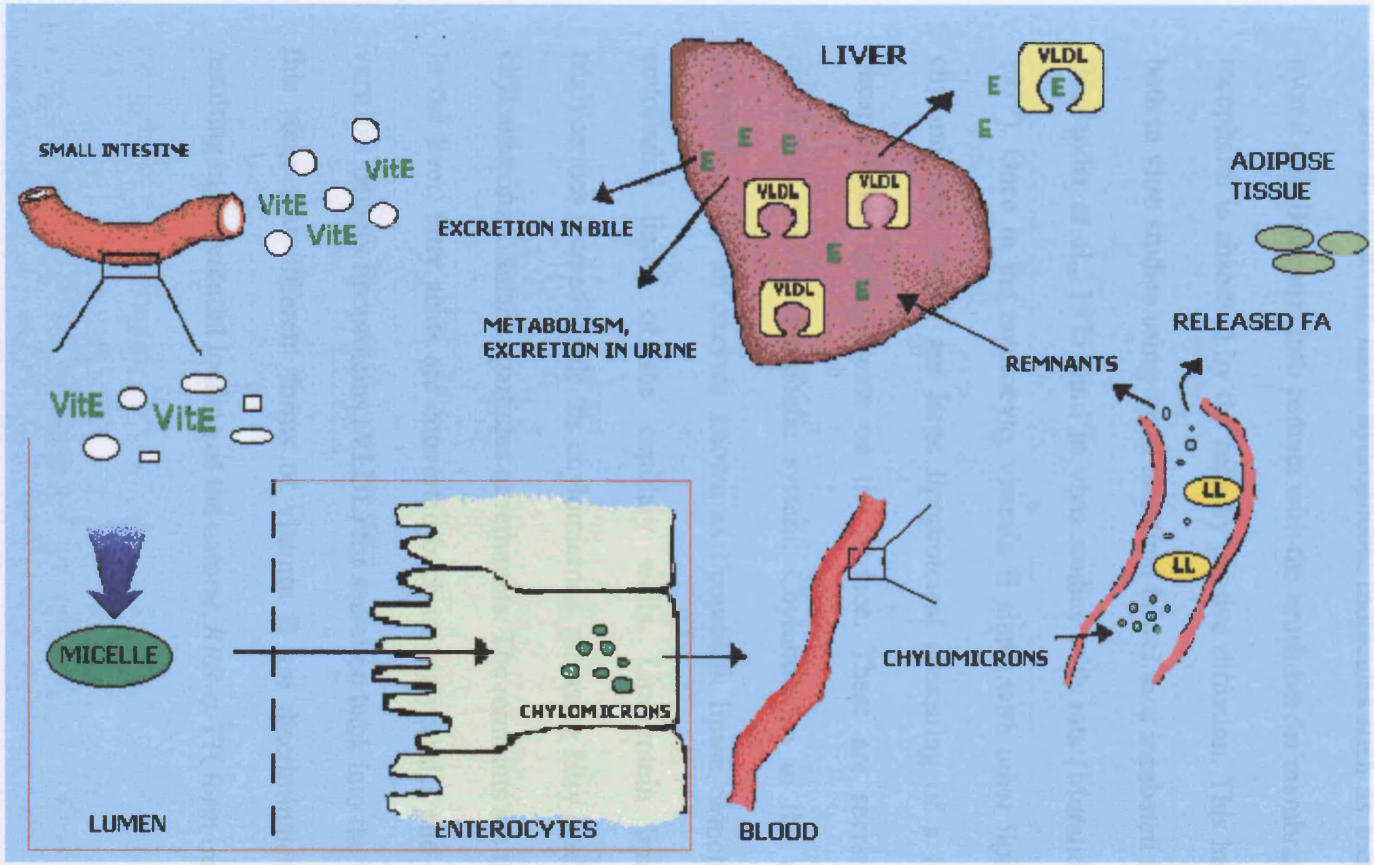


Figure 1.4; Vitamin E absorption and its transport through the body

[VitE= vitamin E; LL= lipoprotein lipase ; VLDL= very low density lipoprotein; FA= fatty acid]

hydrolyses triglycerides in the small intestine to monoglycerides and fatty acids. The products of lipolysis form molecular aggregates with bile salts called micelles, which are able to solubilise more hydrophobic molecules such as tocopherol. When these mixed micelles come into contact with the brush-border membrane of the enterocytes, tocopherol is absorbed by the cells by passive diffusion. This has been confirmed by both in vitro studies using small intestinal bowel sacs incubated in a micellar medium (Hollander et al., 1975) and in vivo studies with rats (Muralidhara and Hollander, 1977). Once in the enterocyte, vitamin E along with other lipids, is packaged into chylomicrons, which are large lipoproteins, consisting of a lipid-rich centre and phospholipids and proteins on the surface. These are secreted into the plasma circulation via the lymphatic system. Chylomicrons in the circulation are then metabolised by an enzyme known as lipoprotein lipase, which is anchored in the endothelial lining of the capillary walls. Lipoprotein lipase hydrolyses the triglycerides located inside the chylomicrons, releasing fatty acids and converting the chylomicrons to smaller particles or remnants. The remnants are taken up by the liver. In the liver, fatty acids are converted back to triglycerides and loaded onto nascent very low density lipoproteins (VLDL) for secretion back into the blood stream. It is at this stage that different forms of vitamin E are discriminated (see section 1.5), resulting in preferential loading of the natural, *RRR*-α-TH form onto VLDL.

1.5 Regulation of vitamin E uptake and tissular distribution

1.5.1 Introduction

Studies in man have shown that upon ingestion of equal doses of deuterium labeled *RRR* and *SRR*-α-TH, similar amounts of both isomers appear in the chylomicrons, suggesting comparable intestinal absorption (Traber et al., 1990). However, many studies have demonstrated that the natural *RRR*-α-TH form is preferentially enriched in VLDL, with subsequent enrichment of all circulating lipoproteins with this form (Traber et al, 1990; Traber, 1994). Similar experiments using deuterated labeled compounds have investigated the uptake of the synthetic *SRR*-α-TH isomer, and natural *RRR*-α-TH isomer in the plasma and different tissues of male Sprague-Dawley rats (Ingold et al., 1987). The synthetic α-TH, *SRR*-isomer, was found to accumulate in the liver. In all other tissues and the plasma, however, there was an excess of the natural, *RRR*-form. These findings were later confirmed by Traber et al (1998) where human volunteers were given a supplement of 150mg of deuterated d_6-all-rac-α-tocopheryl acetate and d_3-*RRR*-α-tocopheryl acetate over a twenty four hour period. Natural *RRR*-α-TH was taken up twice as efficiently as synthetic *SRR*-α-TH into the plasma. It was later confirmed by radio-labeling experiments carried out by Kaneko et al (2000) that the 2*R* configuration was preferentially secreted back into the plasma circulation via VLDL. Similar experiments were carried out to compare α-TH with γ-

TH, where it was found that upon supplementation with labeled α-TH, the newly absorbed α-TH replaced unlabeled plasma α-TH and γ-TH. Another study showed that despite γ-TH intake exceeding that of α-TH in a typical American diet, plasma levels of γ-TH in fasting subjects were usually <20% of α-TH (Swanson et al., 1999). Although α- and γ-TH exhibit similar absorption efficiencies, the overall retention of γ-TH and secretion back into the plasma circulation after reaching the liver is considerably less than that for α-TH. It therefore appears that there is a mechanism that specifically selects 2*R* forms of α-TH for secretion back into the plasma circulation.

Due to thermodynamic barriers imposed on the diffusion of hydrophobic molecules through an aqueous environment, there exist specific binding proteins that facilitate lipid transport. These binding proteins recognize a specific lipid with high affinity and then release the ligand to an acceptor membrane. There are several lipid binding protein families. One of these families consists of proteins with a conserved lipid-binding domain, a CRAL-TRIO motif (also known as Sec14 domain). This family includes cellular retinaldehyde binding protein (CRALBP), yeast phosphatidylinositol transfer protein SEC14p, supernatant protein factor (SPF, also known as tocopherol associated protein (TAP)) and α-tocopherol transfer protein (α-TTP).

Three tocopherol regulatory proteins (TRPs) have been identified to date, that specifically bind to THs and all belong to the same family of cytosolic lipid binding and transfer proteins. These proteins include the α-tocopherol transfer protein (α-TTP), tocopherol associated proteins (TAPs) and tocopherol binding proteins (TBPs).

The regulation of TRPs is not yet understood. Because vitamin E deficiency is almost always found during protein calorie malnutrition, it may be that either vitamin E and/or protein deficiency is responsible for the regulation of TRP levels and/or activity. Upon inadequate protein intake, hepatic α-TTP levels are less well maintained compared to other proteins (Tamai et al., 2000). Oxidative stress may also modulate α-TTP. It has been shown that in diabetic rats and in humans, hepatic α-TTP and its mRNA are increased along with plasma α-TH levels (Tamai et al., 2000).

1.5.2 α-Tocopherol transfer protein (α-TTP)

Catignani (1980) identified a 30-35 kDa liver protein in human and rat as α-TTP. Since then, this protein has been reported to be present in rat brain, spleen, lung and kidney (Hosomi et al., 1997) and in human brain (Copp et al., 1999). The relative affinities of purified α-TTP for different THs, have been determined by competition between vitamin E analogues for transfer between liposomes and membranes in vitro and has been reported as follows ; 100% for *RRR*-α-TH, 38% for β-TH, 9% for γ-TH, 2% for δ-TH, 12% for α-tocotrienol and 11% for *SRR*-α-TH (Hosomi et al, 1997). Initially it was postulated that α-TTP actively incorporated α-TH with the 2*R* configuration into VLDL. However, subsequent experiments using brefeldin A, which inhibits VLDL secretion from the Golgi apparatus indicated no affect on the secretion of α-TH by hepatocytes in culture (Arita et al., 1997). This would suggest that α-TH is not transferred to nascent VLDL during its assembly but possibly at a later stage.

Blatt et al (2001), proposed that α-TH efflux from hepatocytes depended on the ability of α-TTP to transfer α-TH to the plasma membrane.

α-TTP is a cytosolic protein which specifically binds α-TH (Panagabko et al., 2002). Recently, Meier and coworkers elucidated the crystal structure of α-TTP, and reported two different conformational states of the molecule (Meier et al., 2003), representing either an open or a closed conformation involved in the TH transfer mechanism. The difference between the open and closed conformations is the positioning of extracellular fluid exposed residues that can be thought of as a "lid", that affects the access of ligands to the lipid binding site (CRAL-TRIO motif) located inside a hydrophobic pocket. In the closed conformation the large hydrophobic area of the lid is in direct contact with the *RRR*-α-TH side chain, whereas on lid opening the hydrophobic residues of the lid shift towards the exterior and inserts into the membrane lipid bilayer. As a result the open lid conformation at the surface of the protein mediates ligand exchange between the hydrophobic cavity of the protein and the acyl-chain environment of the membrane bilayer, with the bound TH ligand being released into the membrane. Conversely, TH is moved into the empty or water-filled binding pocket by lid closure. Upon closure, the lid exposes a more polar face to the extracellular fluid and membrane and the charged carrier ligand complex can leave the membrane

Mutations in the gene encoding α-TTP have been shown to result in a rare inherited disorder termed ataxia with isolated vitamin E deficiency or AVED (Ben Hamida et

al., 1993). AVED patients exhibit normal intestinal α-TH absorption and transport in the chylomicrons but fail to produce VLDL enriched with α-TH, resulting in low RRR-α-TH in plasma and tissues. Untreated patients have a characteristic progressive peripheral neuropathy resulting in ataxia. The biochemical characterisation of six misense mutations in α-TTP that are found in AVED patients was achieved by expression studies in E.coli. These studies suggested that AVED may not arise from an inability of α-TTP to bind or to transfer α-TH, due to the TTP mutations impact in vitro on TTP activity being quite benign, but rather from defects in other biochemical activities of the protein, yet to be described (Morley et al., 2004). Three of the mutations studied, R59W, E141K and R221W, were associated with severe and early onset of AVED, that in vivo demonstrated profound effects and in vitro only a 2-3 fold reduction in α-TTP transfer kinetics. The other mutations were associated with milder forms of AVED, which demonstrated perturbations of TH homeostasis in vivo, but in vitro showed similar activity to wild-type α-TTP.

1.5.3 Tocopherol-associated protein (TAP) and tocopherol-binding protein (TBP)

In addition to α-TTP, there may be other proteins that are responsible for the distribution of α-TH to intracellular compartments and which regulate signal transduction and gene expression by α-TH. The functions of both TAP and TBP remain undefined. These two proteins contain a lipid binding domain, CRAL-TRIO

motif, and are suggested to have a role in the modulation of intracellular rather than tissue distribution of α-TH.

The first discovery of TAPs was that of a 46kDa protein found in bovine liver cytosol (also known as supernatant protein factor (SPF))(Stocker et al., 1999). The human homologue of bovine TAP was later described by Zimmer et al (2000) and termed hTAP. These TAP proteins were shown to stimulate microsomal squalene epoxidase (Shibata et al., 2001) in addition to their ability to bind α-TH (Stocker et al., 1999; Zimmer et al., 2000; Yamauchi et al., 2001). Although no specific function has been demonstrated for this protein, it shows significant homology to α-TTP. hTAP has a carboxy terminus domain, however, which is not present in α-TTP, which suggests these proteins have different functional roles. Additionally TAPs exhibit a GTP binding motive and GTPase activity (Zimmer et al., 2000) which may be important to confer hTAP regulatory properties and modulation of phospholipid/tocopherol secretion and/or phospholipid/tocopherol signalling (reviewed by Azzi et al., 2002). Immunoprecipitation and purification of three recombinant hTAPs (hTAP 1, 2 and 3) indicated that they share high sequence homology suggesting they perform similar functions. These hTAPs also demonstrated binding capabilities not only to THs but also to phosphatidylinositol, phosphatidylcholine and phosphatidylglycerol (Kempna et al., 2003). It has been shown that TAPs bind THs with an equal affinity to phosphatidylinositol (Kempna et al., 2003) which suggests that TAP is non-selective toward THs (Panagabko et al., 2002; Manor and Atkinson, 2003).

TBP, a 14.2kDa cytosolic protein was first identified by Dutta-Roy et al (1993). Purified TBP from rabbit heart cytosol has been found to bind selectively to α-TH, and is capable of increasing in vitro transfer of RRR-α-TH from liposomes to mitochondria by 8 to 10 fold. Dutta-Roy (1994) also described a plasma membrane TBP (TBPpm) in human erythrocytes and liver, that may be responsible for the regulation of α-TH in these cells. α-TH binding sites have also been shown to be present in membranes of different cell types, such as aortic endothelial cells and rat adrenal cells (Kitabchi et al., 1980). The binding affinity of TBPpm in human erythrocytes was similar for α-, β-, δ- and γ-TH (Bellizzi et al., 1997). This may enable the erythrocytes to use all the TH homologues to prevent oxidative damage.

1.6 Vitamin E metabolism in the human body

1.6.1 General metabolism of lipids and xenobiotics

The human body is exposed to a range of different xenobiotics and lipophilic compounds from a variety of pharmaceutical and food components throughout the course of a lifetime. Many of these molecules remain un-ionized or partially ionized at physiological pH in the body. They are often bound to plasma proteins and are not readily excreted in the bile or urine or efficiently reabsorbed. The body has developed specialised mechanisms capable of handling these compounds by either increasing their rate of excretion or by detoxification enzymes (reviewed by Liska, 1998).

Although the underlying metabolic processes are often considered to be detoxification processes, this is not always the case. In many cases the resultant metabolites are either more active and/or toxic than the parent compound. The "detoxifying" mechanisms show great variability depending on environmental responses, lifestyle and genetic background among different individuals. The compound must be rendered water-soluble in order to facilitate excretion as waste products. These metabolic reactions are carried out mainly in two organs, namely the liver and kidney. In the liver the end products are excreted via the bile into the feces and in the case of the kidney excretion occurs in the urine. The first analytical attempts of scientists to understand how the body manages various lipophilic and xenobiotic compounds began by the collection and analysis of urine almost two and a half centuries ago. In 1773 hippuric acid was identified in human urine, but it was not until 1842 that Keller, after ingesting benzoic acid, reported that it was the amount of ingested bezoic acid that was related to hippuric acid excretion. It is now known that hippuric acid comprises benzoic acid conjugated to glycine (Keller, 1842). It was later observed that adminstration of other acids also resulted in the excretion of hippuric acid, which led to the postulation of oxidation of these acids to benzoic acid as an intermediate step.

Lipophilic and xenobiotic compounds initially undergo side-chain shortening by α, ω and β-oxidation pathways located in the ER, mitochondria and peroxisomes. These side-chain shortened products then undergo biotransformation to water-soluble compounds (Williams, 1947). The biotransformation mechanism commonly consists of two steps. Firstly a polar group is introduced to the compound, for example, a

hydroxyl or carboxyl group by reduction/oxidation mechanisms. This principally occurs in the endoplasmic reticulum (ER) and is catalysed by cytochrome P450 and cytochrome P450 reductase supergene family of enzymes with the use of NADPH as a cofactor. The resulting molecules are able to cause damage to proteins, RNA and DNA and, therefore, need to undergo further modification. In the second step, these reactive molecules are conjugated to water-soluble groups, such as glucuronic acid, sulphate, glycine, glutamine, taurine, ornithine and glutathione. Glucuronyl transferase enzymes in the ER, for example, catalyse the binding of glucuronic acid to hydroxyl groups of these reactive molecules. Sulphate conjugation occurs in the cytosol and binds to phenol groups. These polar conjugates may then be readily excreted either in the bile directly from the liver or reabsorbed into plasma and transported to the kidney where they are filtered into the urine.

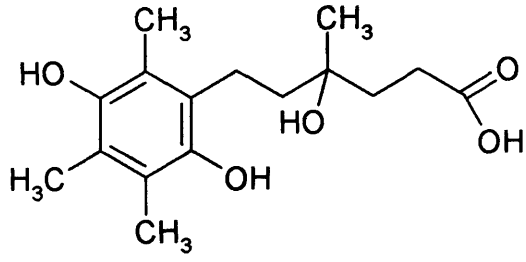
Additionally, the intestine appears to play an important role in metabolism. It contains metabolising enzymes, such as the cytochrome P450 enzyme, CYP3A, at high concentrations. The gut microflora also produce compounds that are able to induce or inhibit these enzyme activities, and are also able to remove some conjugated moieties and convert the compounds to their original form, subsequently allowing re-entry into the circulation.

1.6.2 Vitamin E metabolism

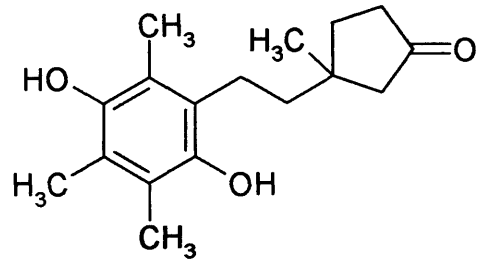
1.6.2.1 Products of vitamin E metabolism

The structure of vitamin E compounds, both tocopherols and tocotrienols, consist of a side-chain and a chromanol ring, which are capable of producing two distinct groups of metabolites. One group of metabolites result from the reaction of the chromanol ring with oxidants, and the other group is produced by shortening of the side chain.

Simon et al (1956) carried out vitamin E supplementation studies in both rabbits and humans. They discovered two urinary metabolites, known as 2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethyl-1,4-benzoquinone (α-tocopheronic acid, α-TA) and its γ-lactone derivative, α-tocopheronolactone (α-TL), now referred to as the 'Simon metabolites' (figure 1.5). The detection of these metabolites appeared to show evidence of oxidative ring opening of the α-TH. It was postulated that the metabolic pathway of TH proceeded by the following steps (see figure 1.6); 1-chroman ring is opened, leading to α-tocopheryl quinone (α-TQ); 2- reduction of the α-TQ to α-tocopheryl hydroquinone (α-TQH_2); 3- side chain shortening by β-oxidation to give α-TA and side-chain rearrangement to form α-TL; 4- conjugation of metabolites with glucuronic acid, and then 5- excretion in the urine. Following oral administration of α-TQ in humans, Schmandke (1965) showed that α-TA was excreted in the urine. The amount of metabolite, however, was only 0.3% of the original dose, which was most likely the result of poor absorption of α-TQ by the intestine.



α-tocopheronic acid (α-TA)



α-tocopheronolactone (α-TL)

Figure 1.5; The 'Simon urinary vitamin E metabolites', α-tocopheronic acid (α-TA) and α-tocopheronolactone (α-TL).

Following injection of rats with radiolabeled δ-TH, a new urinary metabolite was discovered by Chiku et al (1984). The metabolite was identified as δ-carboxy-ethyl-hydroxychroman (δ-CEHC), consisting of an intact chroman ring. It was proposed that this metabolite was formed by the shortening of the phytyl side-chain by β-oxidation. The α-TH homologue of δ-CEHC was later identified in human urine (Schultz et al., 1995) and this metabolite i.e. 2,5,7,8-tetramethyl-2-(2`-carboxyethyl)-6-hydroxychroman (α-CEHC), was demonstrated to be the principal urinary metabolite following supplementation with doses of 50-150mg/day of *RRR*-α-TH. This finding further supported the proposal of side-chain metabolism of α-TH without previous oxidative ring opening (see figure 1.6). Enzymatic studies revealed that α-CEHC was excreted in the urine predominantly as sulphate conjugates (Schultz et al., 1995). Although α-TL was also detected in this study, it appeared to result from the artefactual oxidation of α-CEHC. It was therefore considered that the metabolites detected by Simon and his group were produced by artefactual oxidation during sample processing, as this group used drastic acid hydrolytic deconjugation at an elevated temperature as opposed to the more gentle enzymatic deconjugation (Schultz et al., 1995). However, whether some of the detected α-TL is produced in vivo, is still uncertain.

If α-TL is produced in vivo, concentrations should be raised in the presence of oxidative stress and may be a potential in vivo biomarker. This hypothesis was tested in the present investigation (see chapter 4) by exercise-induced oxidative stress, where human subjects undergoing exercise were studied. In this study urinary concentrations

of α-TL were compared to urinary concentrations of 8-isoprostane, a well recognised biomarker of oxidative stress (see chapter 3).

In 1996, a new endogenous natriuretic factor (a factor controlling the body's pool of extracellular fluid by facilitating urinary excretion of sodium) was isolated and characterised as unconjugated γ-CEHC (γ–carboxyethyl-hydroxychroman), also referred to as LLU-α (Wechter et al., 1996). They proposed that this compound was a metabolite of γ-TH. As mentioned previously, Swanson et al found that although there was a higher intake of γ-TH in a typical north American diet, plasma levels were only 20% that of α-TH, due to preferential secretion of α-TH back into the plasma circulation (Swanson et al., 1999). Urinary analysis, on the other-hand, showed greater concentrations of γ-CEHC than α–CEHC. These results confirmed the preferential release of α-TH from the liver into the circulation and the preferential metabolism of γ-TH and excretion of its products.

In confirmation of a sequential side-chain shortening pathway involved in the metabolism of vitamin E, Parker and Swanson (2000), described the precursor of γ-CEHC, γ–carboxymethylbutyl-hydroxychroman (γ-CMBHC) in human urine and also in HepG2 cell cultures grown on medium supplemented with γ-TH. Pope et al (2002) later unequivocally confirmed the presence of α-CMBHC in human urine by the synthesis of α-CMBHC standard. A precursor of CMBHC with two extra carbons, γ-carboxymethylhexyl-hydroxychroman (γ-CMHHC), was subsequently described by Birringer and coworkers in 2002 in studies with HepG2 cell cultures (figure 1.6) (Birringer et al., 2002).

1.6.2.2 Proposed pathway for the metabolism of vitamin E

Vitamin E metabolism results in the formation of water-soluble metabolites with successive side-chain shortened structures, including CEHC, CMBHC and CMHHC, which are conjugated with glucuronide or sulphate groups and excreted in the urine. This provides evidence of vitamin E undergoing metabolism by a similar mechanism to that involved in the general metabolism of lipids and xenobiotics. The saturated phytyl side-chain of the tocopherols is identical to the structure of a well studied fatty acid (FA), phytanic acid. The metabolism of tocopherol, therefore, might be expected to follow the same chain-shortening β-oxidation pathway as phytanic acid without prior oxidative ring opening (see chapter 5 section 5.1.4 for more detail) (figure 1.6). It is not known whether this takes place in mitochondria and/or peroxisomes. This question will be addressed in studies in patients with mitochondrial and peroxisomal disorders (chapter 5). Additionally the 'Simon metabolite' α-TL may be formed upon oxidative ring opening followed by β-oxidation (forming α-TA) and finally side-chain rearrangement to form a lactone group.

β-Oxidation

As discussed above, analysis of rat and human urinary vitamin E metabolites and studies in tissue culture using HepG2 cells have shown the secretion of successive side-chain shortened metabolites, CMBHC and CEHC (figure 1.6). The detection of these metabolites confirms that TH undergoes systematic side-chain shortening which may involve β-oxidation.

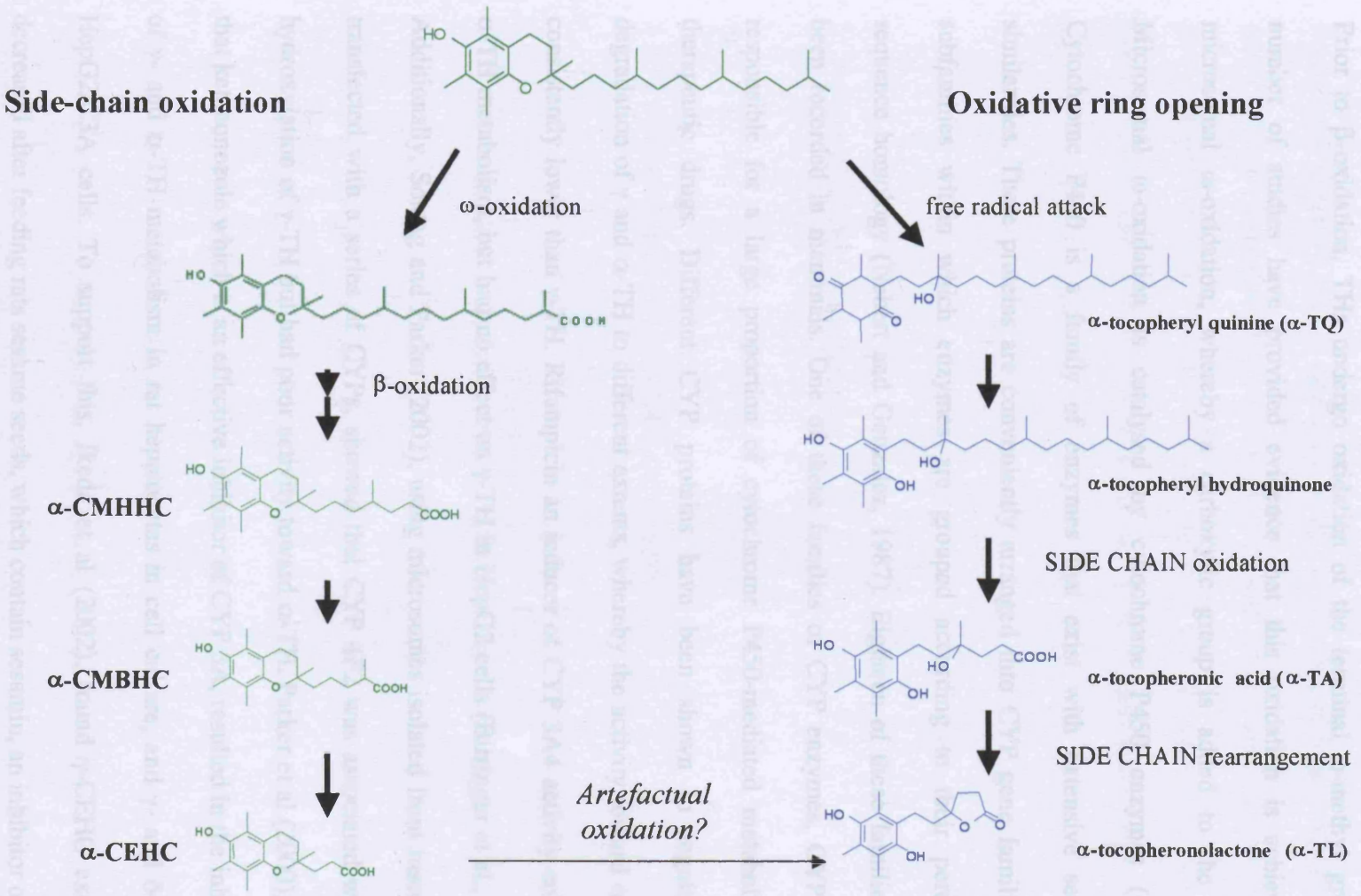


Figure 1.6; The proposed metabolism of vitamin E by side-chain oxidation and oxidative ring opening. [CMHHC=carboxymethylhexyl-hydroxychroman; CMBHC=carboxymethylbutyl-hydroxychroman; CEHC= carboxyethyl-hydroxychroman]

ω-Oxidation

Prior to β-oxidation, THs undergo oxidation of the terminal ω-methyl group. A number of studies have provided evidence that this oxidation is achieved by microsomal ω-oxidation, whereby a carboxylic group is added to the ω-site. Microsomal ω-oxidation is catalysed by cytochrome P450 enzymes (CYPs). Cytochrome P450 is a family of enzymes that exist with extensive sequence similarities. These proteins are conveniently arranged into CYP gene families and subfamilies within which enzymes are grouped according to their percentage sequence homology (Nebert and Gonzalez, 1987). Eighteen of these families have been recorded in mammals. One of these families of CYP enzymes, CYP 3A is responsible for a large proportion of cytochrome P450-mediated metabolism of therapeutic drugs. Different CYP proteins have been shown to regulate the degradation of γ and α-TH to different extents, whereby the activity toward α-TH is consistently lower than γ-TH. Rifampicin an inducer of CYP 3A4 activity enhanced α-TH metabolism, but had no effect on γ-TH in HepG2 cells (Birringer et al., 2001). Additionally, Sontag and Parker (2002), using microsomes isolated from insect cells transfected with a series of CYPs, showed that CYP 4F2 was associated with the hydroxylation of γ-TH but had poor activity toward α-TH. Parker et al (2000), found that ketoconozole which is an effective inhibitor of CYP 3A, resulted in the inhibition of γ- and α-TH metabolism in rat hepatocytes in cell culture, and γ- and δ-TH in HepG2/C3A cells. To support this, Ikeda et al (2002), found γ-CEHC excretion decreased after feeding rats sesame seeds, which contain sesamin, an inhibitor of CYP 3A.

The intracellular site of vitamin E side-chain shortening, however, remains unresolved. Chapter 5 describes two studies that were carried out to investigate the intracellular site of vitamin E side-chain oxidation. The first of these studies involved urinary vitamin E metabolite analysis in patients with mitochondrial and peroxisomal disorders (section 5.2). The second study involved in vitro tissue culture methods using skin fibroblasts and HepG2 cells with and without modulators of peroxisomal and mitochondrial function.

1.7 Functions of vitamin E

1.7.1 Antioxidant properties of vitamin E

1.7.1.1 Introduction to oxidative stress and antioxidant defences

Oxygen and nitrogen are major constituents of the environment in which aerobes exist, and are potentially poisonous molecules. Both oxygen and nitrogen readily react to form highly reactive oxygen and nitrogen species (ROS and RNS) in biological systems. Reactive oxygen species (ROS) include hydrogen peroxide (H_2O_2), and oxygen derived free radicals such as superoxide ($O_2^{\bullet -}$) and the hydroxyl radical ($OH^{\bullet}$) (Halliwell and Gutteridge, 1999). Reactive nitrogen species include nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$). Free

radicals are molecular entities, which contain one or more unpaired electrons in the outer orbit, which make them highly reactive. They can react by either donating or removing an electron from other molecules, which subsequently become radicals themselves, thus propagating a chain-reaction.

Reactive oxygen and nitrogen species are formed during normal cell metabolism and other biological processes, such as a by-product of molecular oxygen flux through the mitochondria, the respiratory burst of macrophages against invading pathogens, and exposure to a range of external influences, such as pollutants, heavy metals and ionising radiation. These reactive species attack all biomolecules of the cell, including lipids, DNA, carbohydrates and proteins. Living organisms have evolved antioxidant defence mechanisms to protect against ROS (Halliwell and Dizdaroglu, 1992). These antioxidant defences include 1)- the catalytic removal of ROS, by enzymes such as superoxide dismutase, catalase and glutathione peroxidase. 2)- minimising the availability of pro-oxidants, such as iron and copper ions by action of proteins e.g. ferritin (iron), transferrin (iron) and ceruloplasmin (copper). 3)- the protection of biomolecules against damage by action of proteins, such as heat shock proteins. 4)- scavenging of ROS by low-molecular-mass species, such as glutathione, ascorbate, uric acid which are water soluble, and α-TH which is lipid soluble. The level of activity of these different antioxidant defence mechanisms varies between different organs, tissue types, cell types and extracellular fluid. In healthy organisms there is a balance between the production of ROS and the antioxidant defence systems. When antioxidant levels, however, are outweighed by ROS and RNS the system is said to be in a state of oxidative stress. Oxidative stress is associated with many disease

pathologies (see chapter 3) and can lead to adaptation by up-regulation of the antioxidant defences, and/or cell injury.

1.7.1.2 Lipid oxidation, vitamin E and redox cycling

The oxidation of lipids, also referred to as lipid peroxidation, by oxygen in the atmosphere is the reason fats become rancid. Lipid peroxidation (figure 1.7) occurs via a chain of reactions that comprise three distinct stages; initiation, propagation and termination (Halliwell and Gutteridge, 1985). Lipid peroxidation is initiated when FAs are attacked by free radical species such as hydroxyl radicals ($^{\bullet}OH$), that are capable of abstracting a hydrogen atom from a methylene group ($-CH_2-$) adjacent to a carbon-carbon double bond. This results in the formation of a carbon centred free radical ($L^{\bullet}$) which undergoes molecular rearrangement and can react with molecular oxygen to produce lipid peroxyl free radicals ($LOO^{\bullet}$). Peroxyl free radicals are sufficiently reactive to abstract further hydrogen atoms from adjacent polyunsaturated acyl chains, resulting in the formation of another lipid peroxyl free radical ($LOO^{\bullet}$) and a stable lipid hydroperoxide (LOOH). This is referred to as the propagation stage, as this secondary radical can lead to further peroxidation. Termination occurs when either two radicals react to form stable products or a compound donates a hydrogen atom to the lipid peroxyl radical, again producing a non-radical product and therefore preventing further peroxidation.

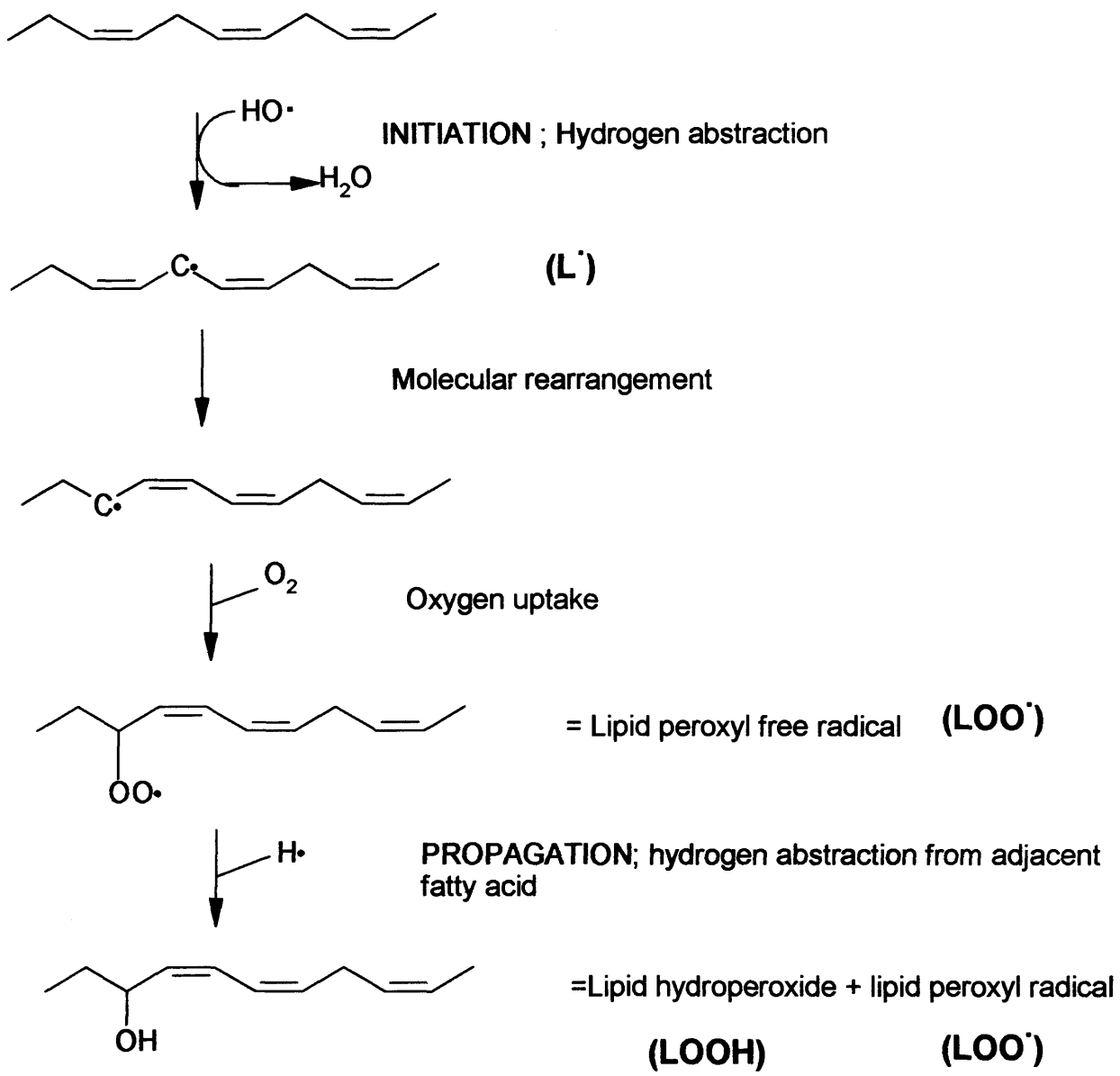


Figure 1.7; Lipid peroxidation

TH is able to interfere with the free radical propagation steps by scavenging FA peroxyl free radicals (L-OO$^{\bullet}$) and thereby terminating the chain reaction of lipid peroxidation. It donates the labile hydrogen atom of its phenol group to the lipid peroxyl radical, thereby forming a lipid hydroperoxide and a tocopheroxyl radical (T-O$^{\bullet}$) (Boguth and Niemann, 1971; Mukai et al., 1982). The reaction with TH is favoured, as the rate of reaction of TH with peroxyl radicals is greater than the reaction with LH. This may be due to the tocopheroxyl radical (T-O$^{\bullet}$) being more stable because of resonance stabilisation, and the possibility of the tocopheroxyl radical being recycled back to TH by vitamin C, which has been shown to occur in vitro (Packer et al., 1979; Niki et al., 1982). The tocopheroxyl radical is stable due to resonance stabilisation of the chroman ring by electron delocalisation (Burton et al., 1985), and is therefore less likely to propagate the radical chain reaction (Boguth and Niemann, 1971; Mukai et al, 1982).

For TH to be an effective in vivo antioxidant, redox cycling must occur to prevent the potential prooxidant activity of the tocopheroxyl radical (T-O$^{\bullet}$). Two different mechanisms have been proposed for TH redox cycling. The 'one electron redox cycle' involves T-O$^{\bullet}$ being reduced back to TH by co-antioxidants in vivo, such as vitamin C, ubiquinol and NAD(P)H dependent enzymes (Packer et al., 1979; Halliwell et al., 1992). T-O$^{\bullet}$ radicals which do not undergo this reduction are proposed to take part in a 'two electron redox cycle'. This involves reaction with further peroxyl radicals in two different reaction pathways yielding two groups of "non-

radical" adducts. Such non-radical adducts include 8α-substituted tocopherones (Winterle et al., 1984; Yamauchi and Matsushita, 1979; Durckheimer and Cohen, 1964; Liebler and Burr, 1992) and epoxytocopherones. To maintain the redox cycle, 8α-substituted tocopherones may be reduced back to TH by enzymatic catalysis (Liebler et al., 1989) or alternatively, α-TQ has also been shown to undergo a reduction reaction to form α-tocopherol hydroquinone (α–TQH_2)(Bindoli et al., 1985; Kohar et al., 1995). α-TQH_2 has been shown to provide potent antioxidant protection (Liebler and Burr, 2000).

1.7.1.3 Antioxidant ability of vitamin E

The heterocyclic chromanol ring of the tocopherols and the tocotrienols is responsible for the antioxidant ability of these compounds. As described above (1.7.1.2), the oxidation of the chromanol ring occurs by the donation of a phenolic hydrogen to a lipid peroxyl radical, forming a resonance stabilised chromanoxyl radical. It would be expected that α-TH would be a more potent hydrogen donor due to its two ortho-methyl groups, compared to β, γ, δ-THs (refer to figure 1.2). Some studies, however, have shown α-TH to be a less potent antioxidant then γ-TH in vitro (Gottstein and Gosch, 1990).

The tocotrienols have been shown by electron spin resonance (ESR) studies to generate tocotrienoxyl radicals (Goh et al., 1990). The three double bonds in the side

chain, however, seem to render the tocotrienols less efficient as in vivo antioxidants in comparison to the tocopherols.

When considering the relative antioxidant activities of the different vitamin E species in vivo, it is not only important to consider their chemical reactivity but also their relative bioavailabilities and biokinetics i.e. association with proteins such as α-TTP.

1.7.1.4 Vitamin E as an antioxidant supplement

Oxidative stress can result from a number of physiological conditions and pathological states including exhaustive exercise (Banerjee et al., 2003), atherosclerosis (Holvoet and Collen, 1994), diabetes (Baynes, 1991) Alzheimer's disease (Pratico et al., 2000a), Parkinson's disease (Ebadi et al., 1996; Betarbet et al., 2000), Down's syndrome (Kedziora and Bartosz, 1988) and environmental factors, such as smoking and inhalation of fumes (Petruzzelli et al., 2000). Vitamin E supplementation studies in individuals undergoing intense exercise or patients with diseases associated with oxidative stress have provided mixed results. Some studies show a beneficial effect of vitamin E supplementation, which is reflected in improved outcome measures or levels of oxidative stress biomarkers. This has been documented in studies in Alzheimer's disease (Sano et al., 1997; Engelhart et al., 2002), type 1 and type 2 diabetes (Skyrme-Jones et al., 2000), hypercholesteroleamia (Neunteufl et al., 1998), coronary heart disease and atherosclerosis (Stephens et al., 1996; Boaz et al., 2000). Hartmann et al (1995) reported that supplementation with vitamin E

inhibited exercise induced DNA strand breakage. Some other studies have however shown that vitamin E supplementation has no beneficial effect, for example in patients with diabetes (Gazis et al., 1999), hypercholesterolaemia (Gilligan et al., 1994) and atherosclerosis (Marchioli, 1999; Yusuf et al., 2000). The lack of consistency in clinical trials with the same condition may be a result of a number of other factors including the effect of combination therapy, stage of advancement of the disease and the dose and duration of vitamin E administered.

1.7.2 Non-antioxidant functions of vitamin E

With the exception of α-TH, the tocopherols and tocotrienols are not retained in humans. α-TH is uniquely recognised and appears to have a number of functions in vivo that are distinct from its antioxidant or radical scavenging abilities. A number of biomolecules that exhibit antioxidant properties also have additional properties that are often responsible for their principal functions i.e. retinol (Tesoriere et al., 1993; Tesoriere et al., 1996) and melatonin (Reiter et al., 1994; Reiter, 1995; Konturek et al., 1997).

Reported functions of α-TH that are not related to its antioxidant activity include effects on the regulation of phosphorylation/dephosphorylation of proteins and cell signalling, gene transcription, anti-inflammatory properties, regulation of cell adhesion processes and regulation of drug metabolism pathways.

α-TH, but not the other tocopherols, has been shown to regulate protein kinase C (PKC) in different cell types, including monocytes (Devaraj et al., 1996; Rattan et al., 1997), macrophages (Freedman et al., 1996), neutrophils (Kanno et al., 1995; Kanno et al., 1996), fibroblasts (Hehenberger and Hansson, 1997) and mesangial cells (Studer et al., 1997; Tada et al., 1997). The mechanism by which PKC activity is inhibited by α-TH is associated with its dephosphorylation via the activation of protein phosphatase type 2A (PP_2A)(Hansra et al., 1996). PKC consists of a family of at least 12 isoforms, which are involved in transmitting signals from the extracellular medium to the nucleus mainly via its ability to activate the transcription factor AP1 (Angel et al., 1987; Schonthal et al., 1988). The age dependent increase of PKC-α is paralleled by increased expression of enzymes that are under the control of PKC via AP1 such as collagenase, which is responsible for the degradation of collagen (Ricciarelli et al., 1999). It has, therefore, been proposed that α-TH may protect skin from age-related collagen breakdown.

α-TH reduces the increased expression by oxidised LDL (oxLDL) of the class A scavenger receptor, SR-A, and the class B scavenger receptor, CD36 (Teupser et al., 1999; Devaraj et al., 2001b). Scavenger receptors (SRs) are responsible for the mediation of cellular uptake of modified lipoproteins such as oxLDL, which are assumed to be involved in foam cell formation in atherosclerosis. The inhibitory mechanism of α-TH is non-antioxidant dependent as other antioxidants, including β and γ-TH failed to decrease CD36 expression. Inhibition is also PKC independent as demonstrated by the lack of effect of PKC inhibitors. Hence, α-TH reduces the uptake

of oxidised lipoproteins with consequent reduction in foam cell formation in atherosclerotic lesions (Ricciarelli et al., 2000).

α-TH also exhibits a role as an anti-inflammatory agent, whereby it inhibits the secretion by macrophages of pro-inflammatory, proatherogenic cytokines such as interleukin 1β (IL-1β) (Akeson et al., 1991; Devaraj and Jialal, 1999) and tumour necrosis factor (TNF-α) (Devaraj and Jialal, 2000; van Tits et al., 2000). The inhibition of the release of cytokine IL-1β has been demonstrated to occur via the inhibition of 5-lipooxygenase (5-LO) activity, by impairment of 5-LO translocation from the cytosol to the membrane by α-TH (Chan et al., 1989; Reddanna et al., 1985).

α-TH has been shown to play a role in the inhibition of monocyte endothelial cell adhesion (Devaraj et al., 1996) by inhibition of counter-receptors on monocytes and inhibition of the transcription factor NF-kB (Islam et al., 1998). NF-kB is responsible for the induction of a variety of genes in atherosclerotic lesions, such as those encoding tumour necrosis factor (TNF-α) and interleukin 1β (IL-1β).

All forms of vitamin E have demonstrated an ability to activate a nuclear receptor, pregnane X receptor (PXR) mediated gene expression in HepG2 cells (Landes et al., 2003) and have therefore been suggested to interfere with drug metabolism via the nuclear receptor, PXR. PXR regulates a variety of drug metabolising enzymes, including cytochrome P450 3A (CYP3A) type enzymes, and is involved in the drug hydroxylation and elimination pathways. As discussed previously, vitamin E appears to be metabolised similarly to xenobiotics in that they undergo ω-oxidation by CYPs

followed by β-oxidation (Birringer et al., 2001; Sontag and Parker, 2002) before being conjugated and excreted in urine or bile. This may suggest that vitamin E forms are able to impose some regulation on their own metabolism.

1.8 Vitamin E-deficiency syndromes

A number of vitamin E deficiency syndromes have been described in human and animal species. Features include foetal resorption, ataxia, growth retardation, encephalomalacia, haematological abnormalities, liver necrosis and myopathy (Goettsch and Pappenheimer, 1931; Wasserman and Taylor, 1972). In man vitamin E deficiency is rare because of the widespread availability of the different forms of vitamin E in the human diet. Typically, vitamin E deficiency results from generalised fat malabsorption but in rare cases it results from a vitamin E specific mechanism as a result of a defective tocopherol transfer protein (α-TTP).

Patients exhibiting severe and chronic vitamin E deficiency as a result of severe fat malabsorption caused by the condition, abetalipoproteinaemia were first described in detail by Muller et al (1977). Abetalipoproteinaemia is an inborn error of lipoprotein metabolism, where infants show undetectable vitamin E concentrations from birth (Muller et al., 1974) and later develop a characteristic neurological syndrome during their second decade of life (Muller et al., 1983) which leads to crippling and blindness. Vitamin E cannot be absorbed or transported in these patients due to a lack

of all lipoproteins except high density lipoproteins (HDL). Severe vitamin E deficiency has also been associated with severe fat malabsorption caused by other factors including obstructive jaundice (cholestatic liver disease) whereby patients lack the necessary bile salts in the intestinal lumen for solubilisation of dietary fats (Harries and Muller, 1971; Elias et al., 1981) and occasionally patients with cystic fibrosis (Elias et al., 1981).

Ataxia with vitamin E deficiency (AVED) is a rare condition resulting from a lack of functional hepatic α-TTP (Ouahchi et al., 1995). As mentioned in section 1.5.2, α-TTP is necessary for the transfer of α-TH to very low density lipoprotein (VLDL) and secretion back into the circulation. In AVED patients, there is an impaired incorporation of RRR-α-TH onto nascent VLDL and impaired secretion of vitamin E back into the circulation and resulting in very low plasma and tissue vitamin E levels. Vitamin E deficiency in AVED patients in the absence of generalised fat malabsorption provided a human condition with a specific deficiency of this vitamin (Sokol et al., 1988). These patients exhibit the same characteristic neurological syndrome as those with generalised fat malabsorption.

In conditions of severe vitamin E deficiency neuropathy is progressive unless treated. Early treatment with α-TH prevents the appearance of neurological signs and symptoms. In cases where symptoms of vitamin E deficiency are already present, treatment halts the progression and in some cases can produce improvement (Muller et al., 1977; Muller et al., 1983). Supplementation of appropriate doses of vitamin E, to patients with vitamin E deficiency or to those at risk of vitamin E deficiency is

recognised as an important preventive measure against the devastating peripheral neuropathy resulting from the deficiency of vitamin E.

The characteristic neuropathy resulting from vitamin E deficiency may be due to the particular susceptibility of the nervous system to oxidative stress. The brain contains high concentrations of polyunsaturated fatty acids (PUFAs) that are susceptible to lipid peroxidation, in addition the brain utilises a large percentage of the body's oxygen and is relatively deficient in antioxidant defense systems. Other functions unrelated to oxidative stress may also be involved.

In the following chapters methods for the estimation of urinary vitamin E metabolites and isoprostanes will be described. Their use in monitoring oxidative stress and the cellular localisation of vitamin E metabolism will be discussed.

Chapter 2

Measurement of urinary vitamin E metabolites

2.1 Measurement of urinary vitamin E metabolites

2.1.1 Introduction

This chapter describes the modification and use of a method initially developed by Pope et al (2000) in our laboratory for the quantitative measurement of urinary vitamin E metabolites using gas chromatography-mass spectrometry (GC-MS). The method was modified in order to reduce artefactual oxidation and shorten the sample preparation time by reducing the incubation period for the enzymatic hydrolysis of the conjugated metabolites. Secondly, the stability of metabolites in urine samples stored under different conditions was tested for both short and long term storage periods.

2.2 Experimental protocol

2.2.1 Introduction to methodology

The protocol used to extract and measure urinary metabolites was based on the methodology developed in our laboratory by Pope et al (2000). The analytical procedure consisted of essentially six steps which included: pre-treatment of urine samples, extraction of metabolites, deconjugation, extraction of deconjugated metabolites, derivatization, and analysis by GC-MS (figure 2.1).

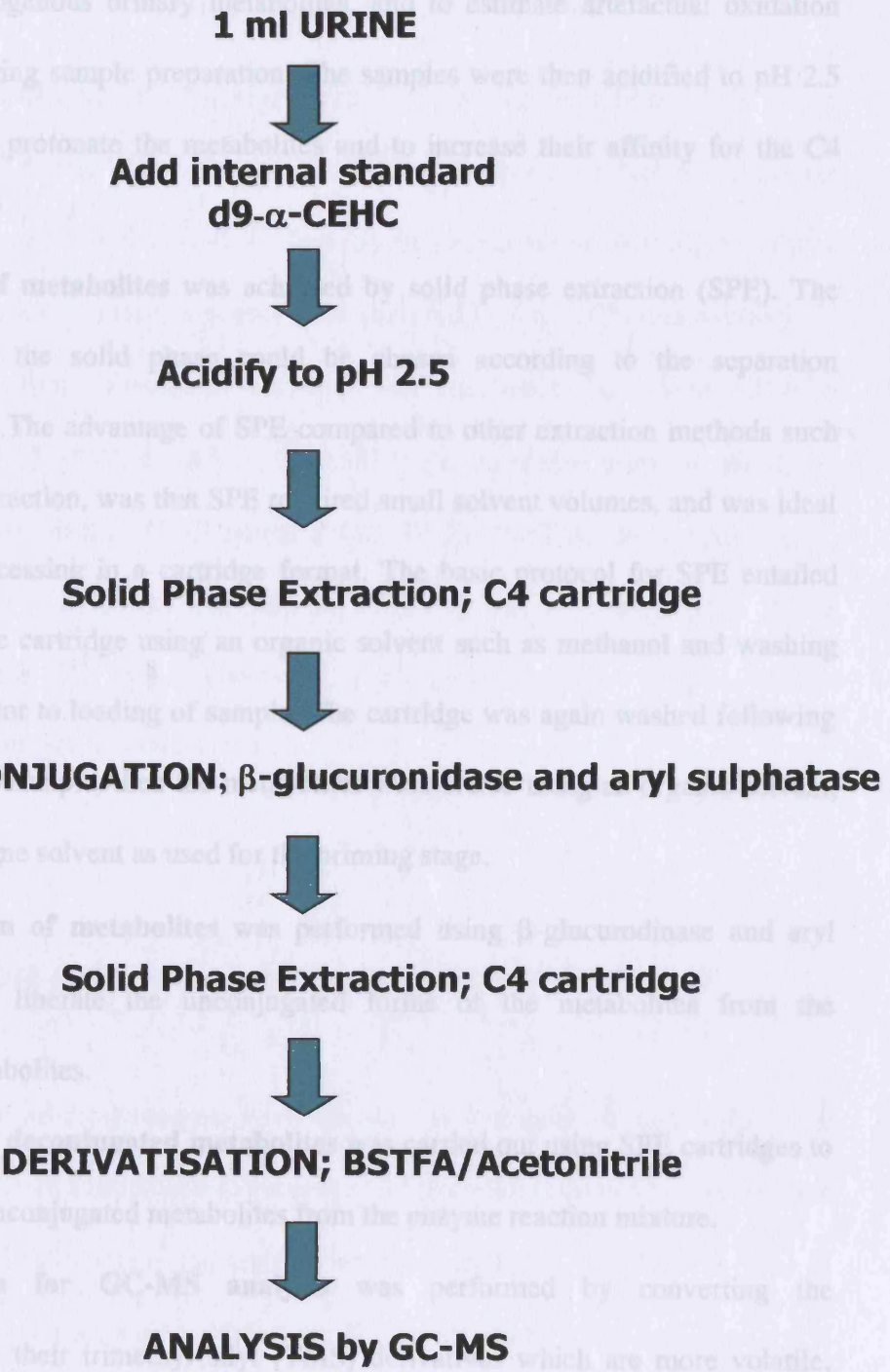


Figure 2.1; Preparation and extraction protocol for urinary analysis of vitamin E metabolites

Pre-treatment involved the addition of standards to the urine samples in order to quantify endogenous urinary metabolites, and to estimate artefactual oxidation occurring during sample preparation. The samples were then acidified to pH 2.5 using HCl to protonate the metabolites and to increase their affinity for the C4 sorbent.

Extraction of metabolites was achieved by solid phase extraction (SPE). The properties of the solid phase could be chosen according to the separation requirements. The advantage of SPE compared to other extraction methods such as solvent extraction, was that SPE required small solvent volumes, and was ideal for batch processing in a cartridge format. The basic protocol for SPE entailed priming of the cartridge using an organic solvent such as methanol and washing with water prior to loading of sample. The cartridge was again washed following loading of the sample, then the metabolites were eluted using an organic solvent, usually the same solvent as used for the priming stage.

Deconjugation of metabolites was performed using β-glucurodinase and aryl sulphatase to liberate the unconjugated forms of the metabolites from the extracted metabolites.

Extraction of deconjugated metabolites was carried out using SPE cartridges to separate the unconjugated metabolites from the enzyme reaction mixture.

Derivatisation for GC-MS analysis was performed by converting the metabolites to their trimethyl silyl (TMS) derivatives which are more volatile, enabling them to be analysed in the gaseous phase by gas chromatography (GC).

Analysis by GC-MS was performed after injecting samples onto the GC column. The GC-MS instrument was programmed to run the sample through a preprogrammed method.

2.2.2 Materials

Trolox was purchased from Sigma-Aldrich Company Ltd and d9-α-CEHC was a gift from Dr G Burton (Steacie Institute of Molecular Sciences, National Research Council, Ottawa, Canada). Isolute C4 solid phase extraction cartridges (500mg sorbent mass, 6ml reservoir volume) were supplied by Jones Chromatography. β-glucuronidase (with sulphatase activity) was purchased from Sigma-Aldrich Company Ltd (G-7017, activity of 100,000 β-glucuronidase units/ml and 7,500 sulphatase units /ml from Helix pomatia type HP-2). The TMS derivatising agent, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce Chemicals Ltd). All other chemicals were purchased from Sigma-Aldrich Company Ltd unless otherwise stated.

2.2.3 Sample preparation and analytical method

Aliquots of 1ml of urine samples were used for the extraction of metabolites. The urine samples were centrifuged to remove solid material, followed by the addition of d9-α-CEHC (10nmoles) and trolox (5nmoles) standards dissolved in 10μl of methanol. The samples were acidified to pH2.5 with 3M HCl and loaded on to C4 SPE cartridges that had been primed using 4ml of methanol, followed by 4ml deionized water acidified to pH2.5. Following the elution of the urine samples, cartridges were washed with 4ml deionized water (pH2.5), to remove any unbound compounds. Elution of the vitamin E metabolites was achieved using 4ml methanol. The cartridges were eluted under gravity, with a constant flow rate

of approximately 0.5 ml/min throughout. The eluates were evaporated to dryness under a stream of N_2, dissolved in 1ml deconjugation solution, which consisted of 500µl deionised water, 475µl 5M sodium acetate (pH 4.5) and 25µl suspension of β-glucurodinase with sulphatase activity (activity of 100,000 β-glucuronidase units/ml and 7,500 sulphatase units/ml from Helix pomatia type HP-2) and incubated at 37°C for 18 hours. At the end of the incubation, samples were again acidified to pH2.5, and extracted using new C4 SPE cartridges. The eluates were evaporated to dryness under a gentle stream of N_2. The unconjugated metabolites were then derivatised to form their TMS derivatives, by redissolving in a derivatising solution consisting of 200µl acetonitrile/BSTFA (1:1), and incubated at 60°C for 60 minutes.

The derivatised samples were analysed by GC-MS. 1µl of derivatised sample was injected by splitless mode onto a HP-1 methyl siloxane column (30m, 0.25mm ID, 0.25µm film thickness) supplied by Jones Chromatography Ltd, in a Hewlett-Packard 6890 series GC system. The GC was linked to a Hewlett-Packard 5973 mass-selective detector (MSD) and Chem station data system. The oven was maintained at 120°C for 2 min at the start of the run. It was then ramped to 200°C, at a rate of 20°C/min, then to 240°C, at 2°C/min. The final ramp was to 300°C at 50°C/min, which was held at 300°C for 5mins. The total run time per injection was 32.2min. The ionization energy was 70 eV, and the MSD was run in SCAN mode to enable detection of the full spectrum of ions.

2.2.4 GC-MS data analysis and metabolite measurement

The internal standards and the metabolites were visualised by extracting chromatograms for the following mass/charge (m/z) ions;

Internal standards- (figure 2.2 and 2.3)

Trolox parent fragment m/z 394, daughter fragments m/z 236/237; d9-αCEHC parent fragment m/z 431, daughter fragments m/z 245/6 and d9-α-TL (oxidised form of d9-α-CEHC standard) parent fragment m/z 431, daughter fragments m/z 318, 245/6.

Metabolites- (figure 2.4 and 2.5)

d0-α-CEHC parent fragment m/z 422, daughter fragments m/z 237; d0-α- TL parent fragments m/z 422, daughter fragments m/z 309, 237 ; d0-α-CMBHC parent fragment m/z 464, daughter fragment m/z 237 and oxidised α-CMBHC parent fragment m/z 626, daughter fragments m/z 536, 309

d0-β-CEHC parent fragment m/z 408, daughter fragment m/z 222/223 ; β-CMBHC parent fragment m/z 450, daughter fragment m/z 2222/23 ; d0-δ-CEHC parent fragment m/z 394, daughter fragment m/z 208/209 ; δ-CMBHC parent fragment m/z 436, daughter fragment m/z 208/209 ; d0-γ-CEHC parent fragment m/z 408, daughter fragment m/z 222/223 ; γ-CMBHC parent fragment m/z 450, daughter fragment m/z 222/223. Chromatographic peaks were identified by the mass spectra, characteristic for each metabolite (figures 2. 2- 2.5). TMS-esters/ethers of α-TL and α-CEHC are isobaric compounds, both showing a large

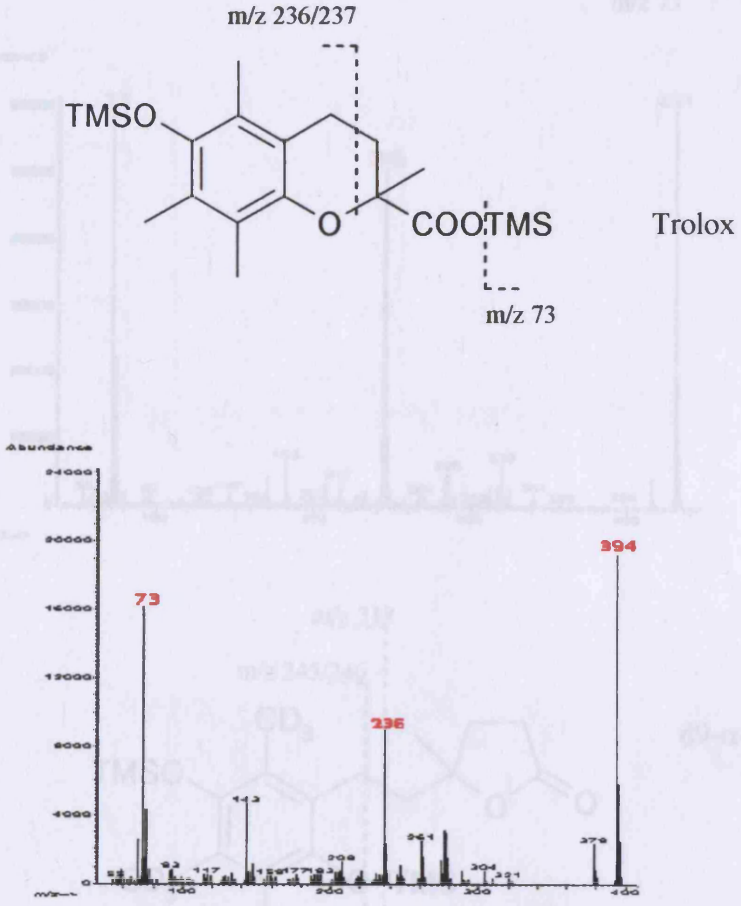


Figure 2.2; Mass spectrum of trolox.

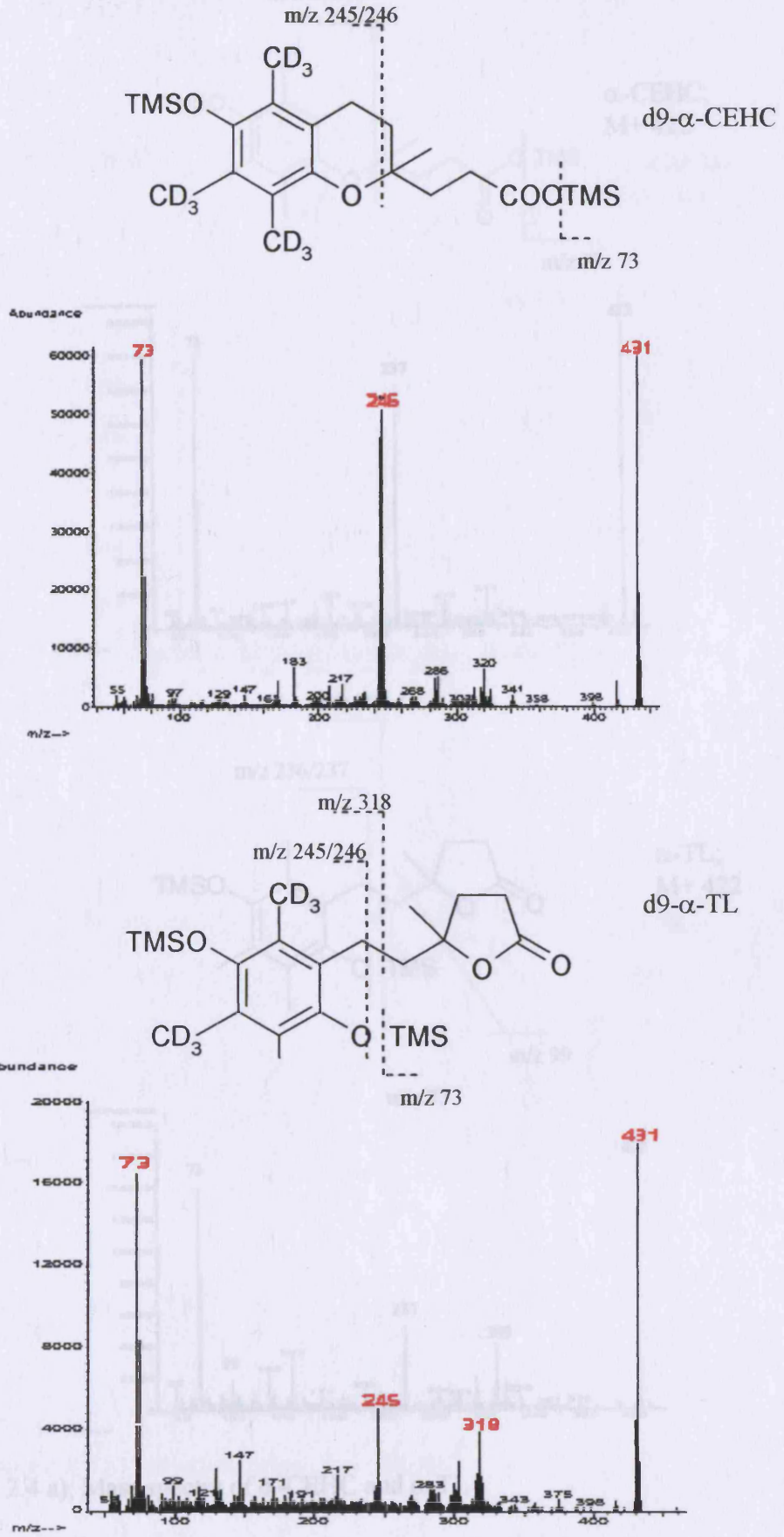


Figure 2.3; Mass spectra of the internal standard d9-α-CEHC and its oxidation product d9-α-TL.

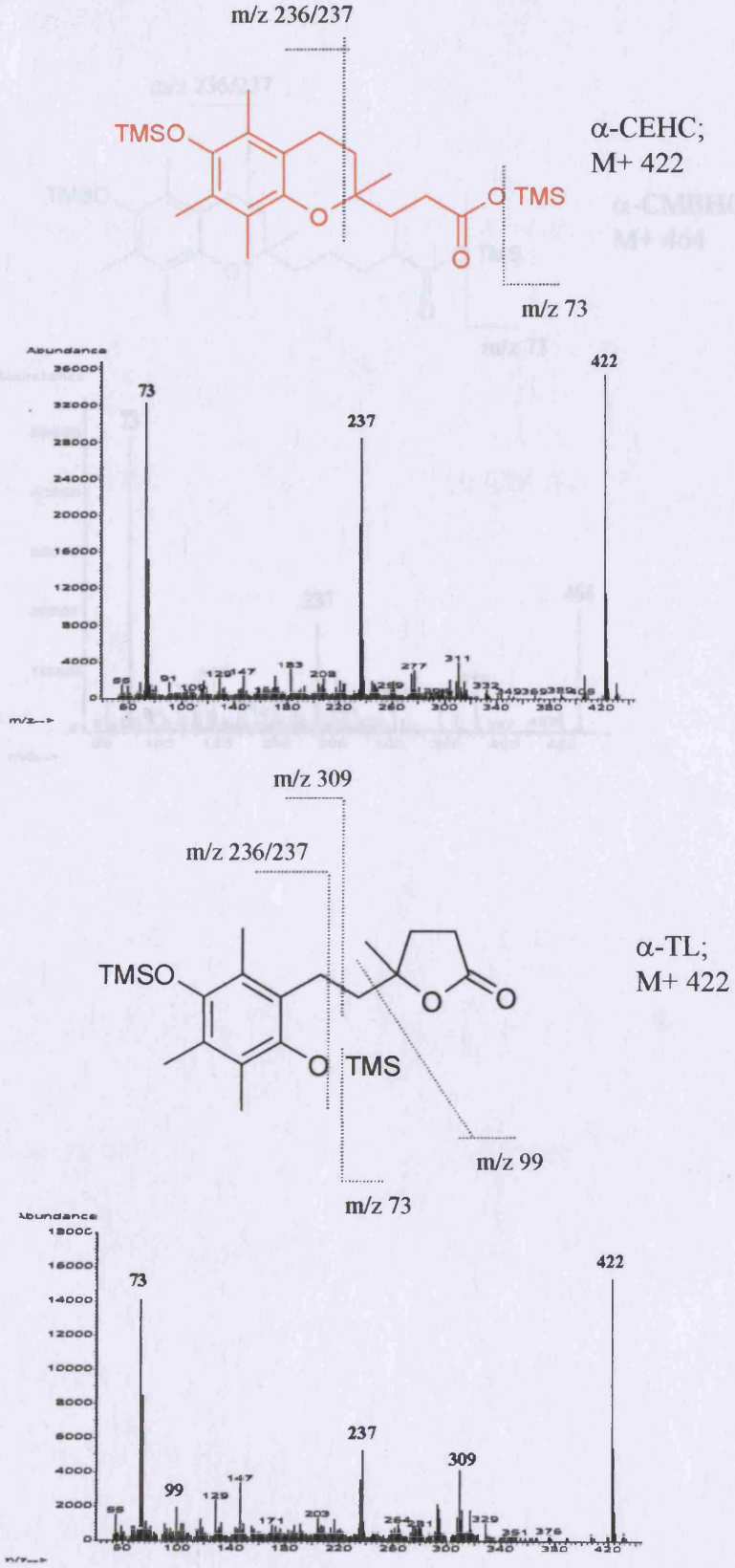


Figure 2.4 a); Mass spectra of α-CEHC and α-TL

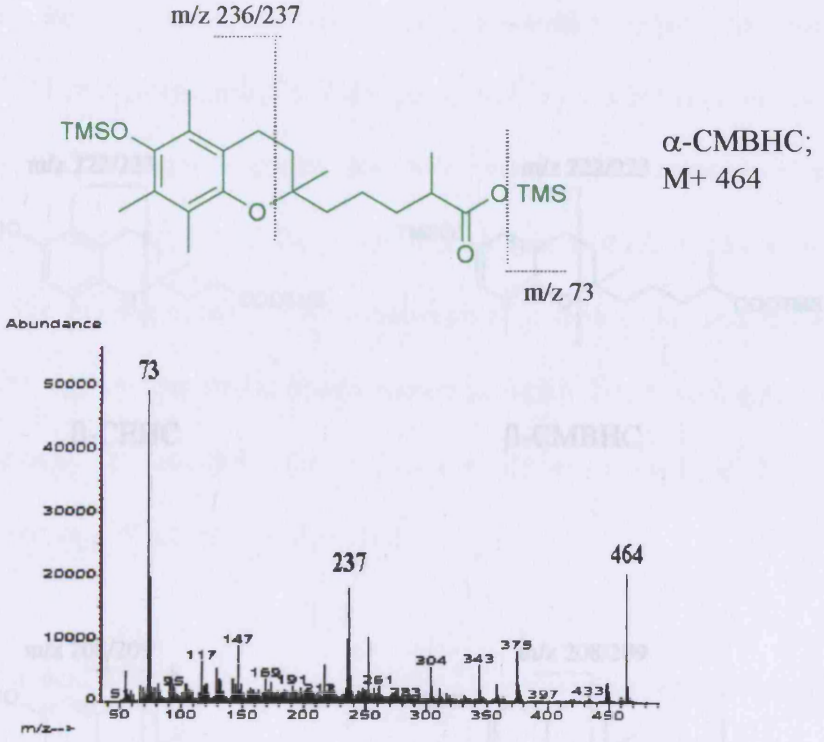
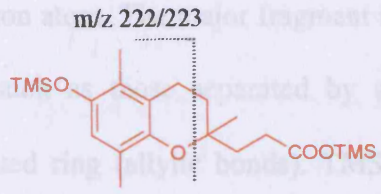
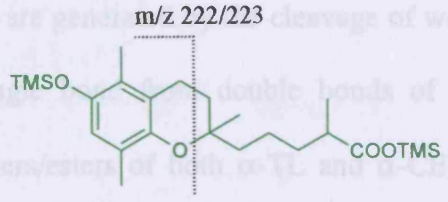


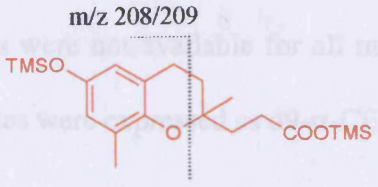
Figure 2.4 b); Mass spectrum of α-CMBHC



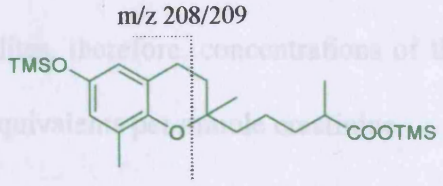
β-CEHC



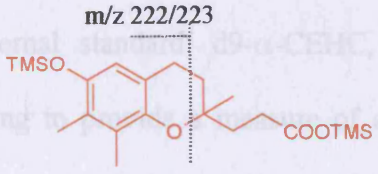
β-CMBHC



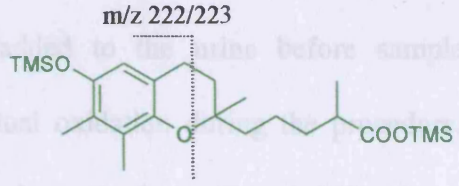
δ-CEHC



δ-CMBHC



γ-CEHC



γ-CMBHC

Figure 2.5; Fragment patterns of β, δ, and γ-tocopherol derived metabolites, CEHC and CMHBCs.

molecular ion of m/z of 422 for the undeuterated metabolite and m/z 431 for the d9-deuterated internal standard. All TMS ethers/esters show characteristic fragments of 73 m/z representing a TMS group that breaks between an oxygen and silicon atom. The major fragment ions are generated by the cleavage of weak bonds, such as those separated by a single bond from double bonds of the unsaturated ring (allylic bonds). TMS ethers/esters of both α-TL and α-CEHC also show cleavage of two allylic bonds shown in figure 2.4, resulting in a m/z 236/237 fragment. In addition, the α-TL also shows a peak at 309 m/z representing cleavage of a single allylic bond.

Standards were not available for all metabolites, therefore, concentrations of the metabolites were expressed as d9-α-CEHC equivalents per mmole creatinine.

2.2.5 The use of d9-α-CEHC as an internal standard

The internal standard, d9-α-CEHC, was added to the urine before sample processing to provide a measure of artefactual oxidation during the procedure. Any d9-α-TL detected must be derived artefactually from d9-α-CEHC (figure 2.6). This was used to estimate and correct for the amount of artefactual oxidation of the endogenous d0-α-CEHC occurring in the individual samples, from the point of spiking to analysis on GC-MS. This presumes that both deuterated (d9) and undeuterated α-CEHC are affected to the same extent by artefactual oxidation.

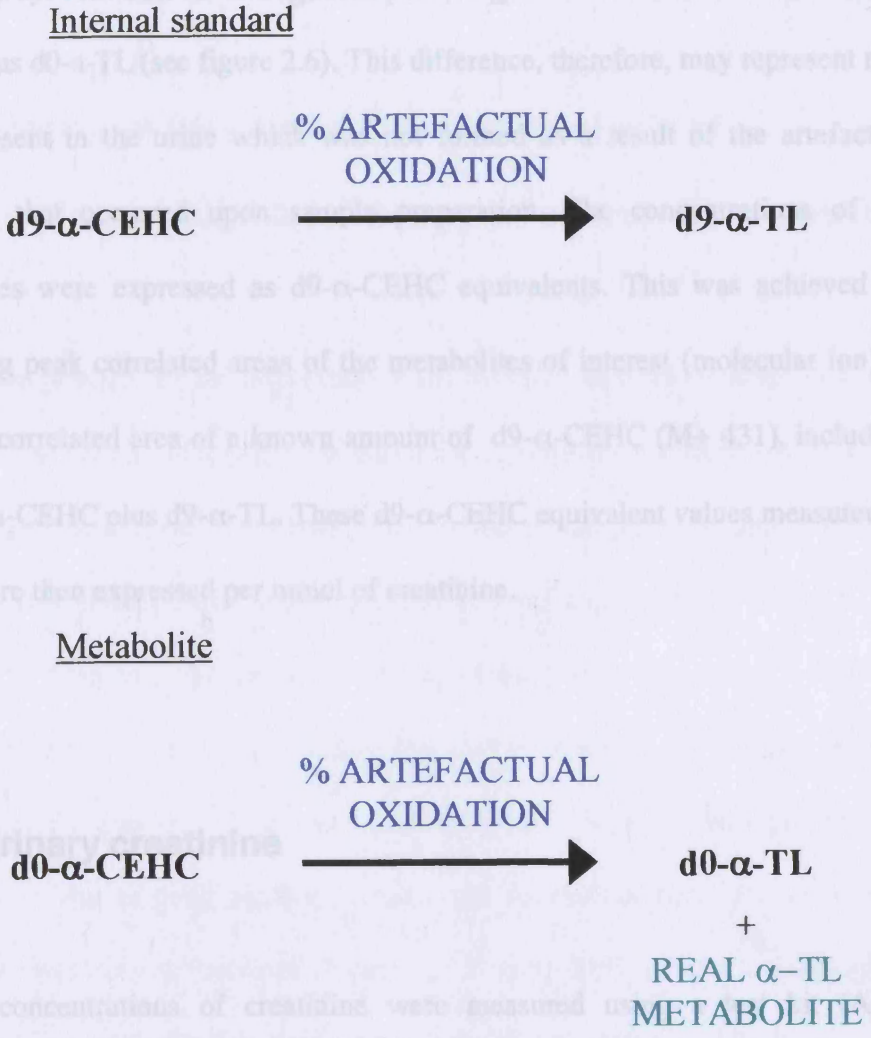


Figure 2.6; The artefactual oxidation of α-CEHC.
The percentage total α-TL detected is almost always greater than that calculated as being due to artefactual conversion from the internal standard. It may therefore be postulated that the difference represents the presence of 'REAL' α-TL in the urine.

The internal standard percentage conversion of d9-a-CEHC to d9-a-TL was almost always less than the endogenous percentage of d0-a-TL in relation to d0-a-CEHC plus d0-a-TL (see figure 2.6). This difference, therefore, may represent real α-TL present in the urine which was not formed as a result of the artefactual oxidation that occurred upon sample preparation. The concentrations of the metabolites were expressed as d9-α-CEHC equivalents. This was achieved by comparing peak correlated areas of the metabolites of interest (molecular ion) to the peak correlated area of a known amount of d9-α-CEHC (M+ 431), including both d9-α-CEHC plus d9-α-TL. These d9-α-CEHC equivalent values measured in moles were then expressed per mmol of creatinine.

2.2.6 Urinary creatinine

Urinary concentrations of creatinine were measured using a test kit (ABX Diagnostics, CREATININE 80) on a COBAS analyser. The test involved the kinetic determination of creatinine, without deproteinisation, by the complex formed by creatinine and picric acid in an alkaline medium (Vasiliades, 1976).

2.3 Modifications of the method

2.3.1 Incubation time for enzymatic hydrolysis of glucuronide and sulphate conjugated vitamin E metabolites

The original protocol for deconjugation of the metabolites from the urine involved enzymatic hydrolysis with β-glucuronidase (with sulphatase activity) at 37^0C for 18 hours. As a result, the preparation and analysis of samples took approximately two days, and resulted in the artefactual oxidation of approximately 10-15 % d9-α-CEHC to d9-α-TL. The aim of these experiments was to examine whether a shorter incubation period would reduce the level of artefactual oxidation without compromising the extent of metabolite deconjugation. Samples were prepared as described in the original method, except the incubation time for enzymatic hydrolysis was varied between 1 and 25 hours. SPE of the unconjugated metabolites was immediately carried out and the eluates evaporated to dryness and stored at 4°C. Once all samples had been extracted they were derivatised and analysed at the same time.

Results and Discussion

The effect of time of incubation on the concentration of vitamin E metabolites and the percentage conversion of d9-α-CEHC to α-TL is shown in figures 2.7, 2.8 and 2.9. Figure 2.7 shows the actual concentrations of α-CEHC and α-TL after correcting for

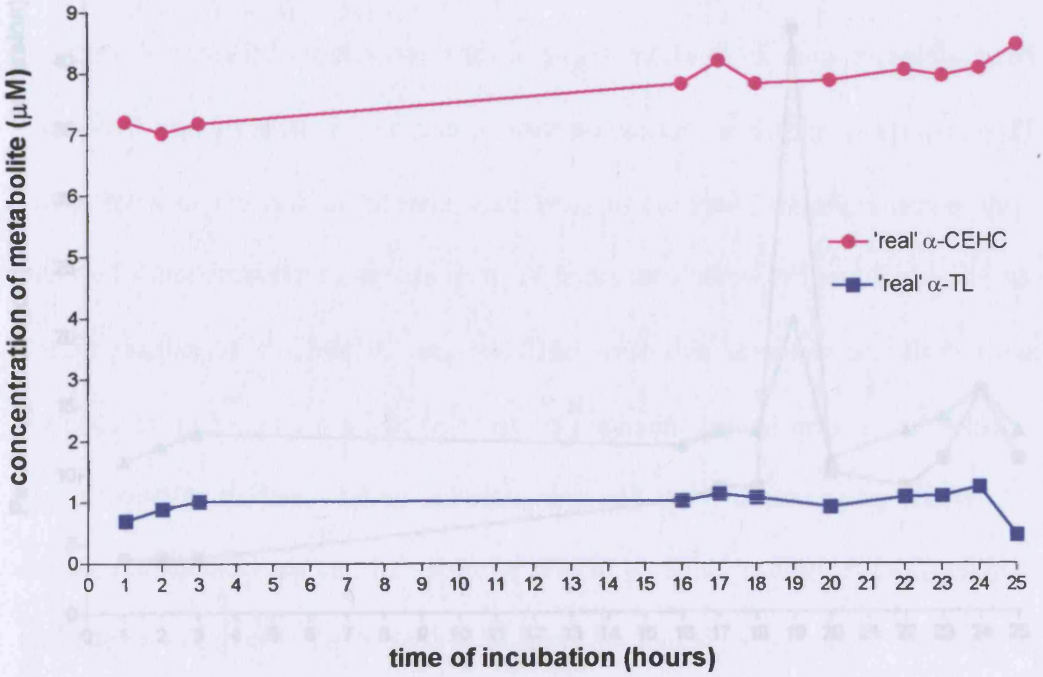


Figure 2.7; Actual concentrations of urinary α-CEHC and α-TL with increasing incubation time of enzymatic hydrolysis

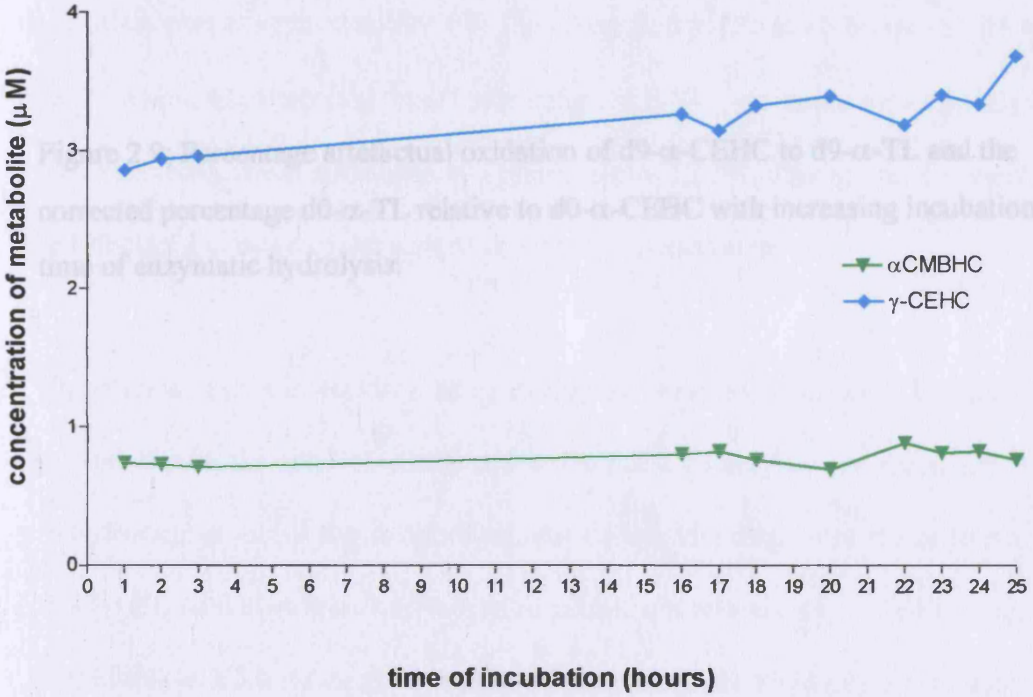


Figure 2.8; Concentration of urinary α-CMBHC and γ-CEHC with increasing incubation time of enzymatic hydrolysis

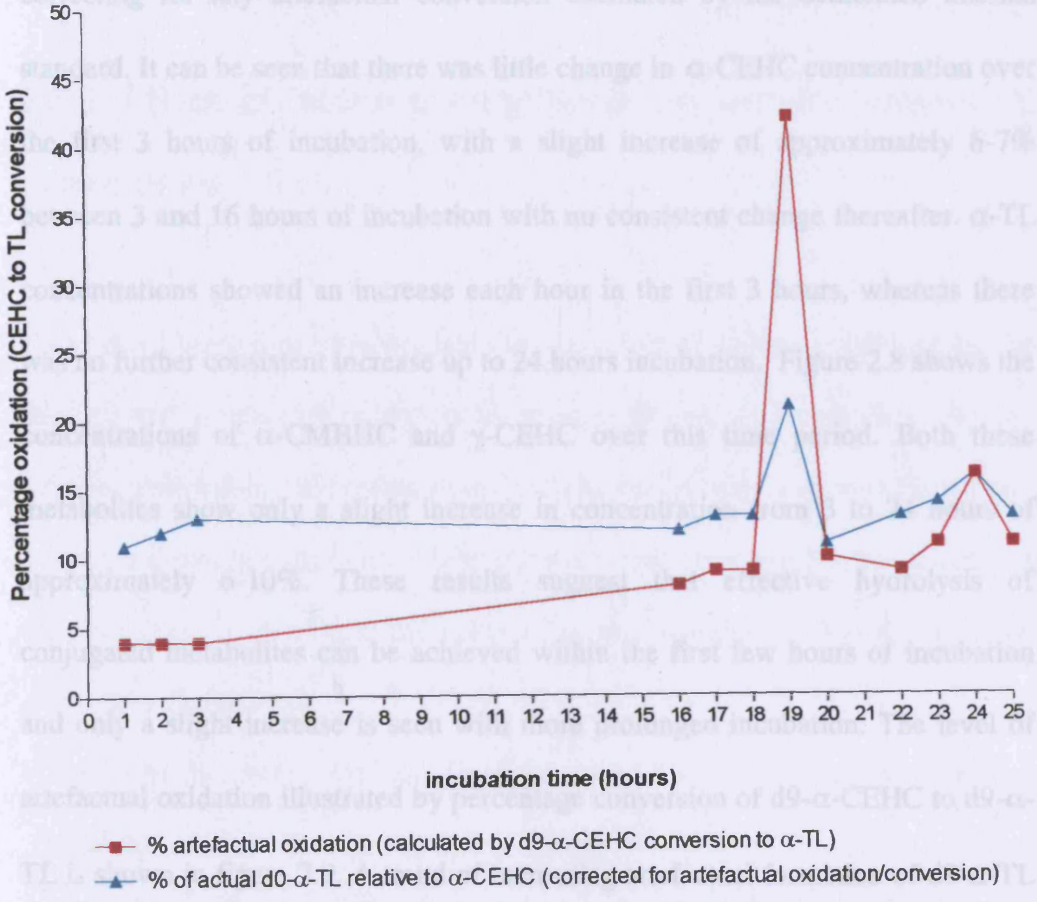


Figure 2.9; Percentage artefactual oxidation of d9-α-CEHC to d9-α-TL and the corrected percentage d0-α-TL relative to d0-α-CEHC with increasing incubation time of enzymatic hydrolysis.

correcting for any artefactual conversion estimated by the deuterated internal standard. It can be seen that there was little change in α-CEHC concentration over the first 3 hours of incubation, with a slight increase of approximately 6-7% between 3 and 16 hours of incubation with no consistent change thereafter. α-TL concentrations showed an increase each hour in the first 3 hours, whereas there was no further consistent increase up to 24 hours incubation. Figure 2.8 shows the concentrations of α-CMBHC and γ-CEHC over this time period. Both these metabolites show only a slight increase in concentration from 3 to 24 hours of approximately 6-10%. These results suggest that effective hydrolysis of conjugated metabolites can be achieved within the first few hours of incubation and only a slight increase is seen with more prolonged incubation. The level of artefactual oxidation illustrated by percentage conversion of d9-α-CEHC to d9-α-TL is shown in figure 2.9. A trend of increasing artefactual formation of d9-α-TL with time is evident. Percentage conversion was constant within the first 3 hours of incubation at approximately 4%. This increased to 8% at 16 hours and 10-11% at 25 hours. The corrected "real" percentage of α-TL (compared to α-CEHC) was relatively consistent with time at approximately 12.5%. The reason for the high artefactual oxidation of the sample at 19 hours is unknown.

From these results it was decided to shorten the incubation period to 3 hours. This would reduce the level of artefactual oxidation with only a very slight effect on the deconjugation of the metabolites. An added advantage was the reduction in analysis time that enabled analysis to be carried out within a day. In all subsequent experiments, a 3 hour incubation period for the enzymatic hydrolysis was used.

2.3.2 Effect of storage conditions on urinary vitamin E metabolites

There was little information available on the effect of storage conditions on the stability and artefactual oxidation of urinary vitamin E metabolites. As the samples analysed in the present study (see chapters 4 and 5) were collected and stored for different periods and under different conditions at other laboratories, it was necessary to investigate the effect of sample storage conditions. The three parameters chosen were;

1) effect of the presence of ascorbate (vitamin C) as an antioxidant to prevent possible artefactual oxidation during storage
2) effect of storage temperature
3) effect of storage time

To investigate the effect of the above parameters on metabolite recoveries, a morning void of fresh urine collected from a 26 year old female colleague (no vitamin supplements consumed for at least 2 years, non-smoker, teetotal), was spiked with internal standards, d9-α-CEHC (10μM) and Trolox (5μM). The mixture was divided into two batches, and ascorbate (5mg/ml) was added to one batch according to our laboratory's standard protocol. From both batches 1ml aliquots were stored at varying temperatures (–80°C to room temperature (21°C)) and analysed at different time points (0-6 months), as indicated in table 2.1. Note that aliquots at time point '0' represent the baseline and are prepared, extracted and analysed within 6 hrs of collection (fresh urine).

STORAGE TIME		STORAGE TEMPERATURE
0 hrs		RT
24 hrs		RT, 4 ^{0}C
48 hrs		RT, 4 ^{0}C
1 wk		RT, 4 ^{0}C, -20 ^{0}C,-80 ^{0}C
2 wks		-20 ^{0}C, -80 ^{0}C
6 wks		-20 ^{0}C, -80 ^{0}C
6 mnths		-20 ^{0}C, -80 ^{0}C

[RT= room temperature]

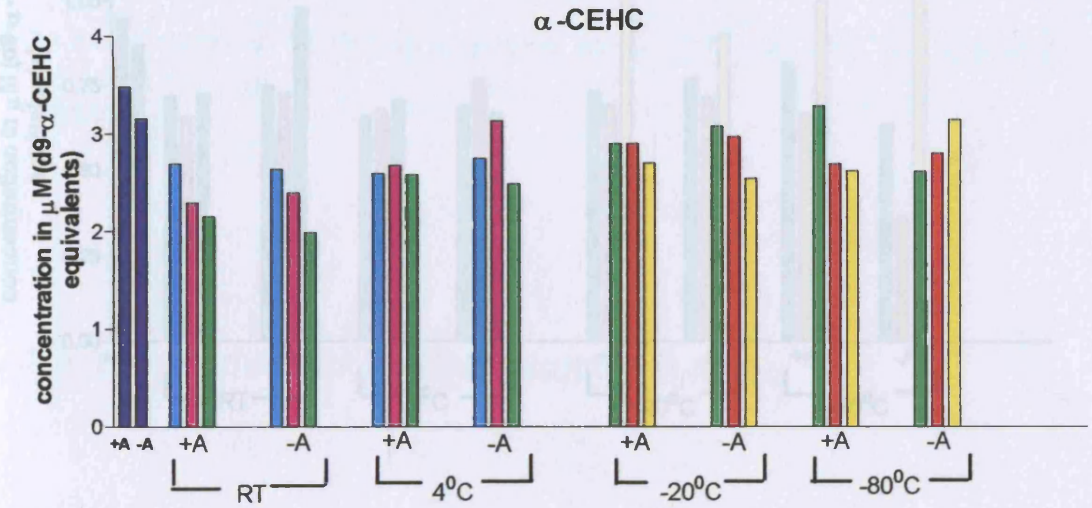
Table 2.1; Plan for the investigation of the stability of metabolites stored at different temperatures for varying lengths of time.

Results and Discussion

The concentrations of α-CEHC and α-TL, for each sample are shown in figure 2.10 and for α-CMBHC and γ-CEHC in figure 2.11. The data show that ascorbate had no consistent significant beneficial effect on the stability or oxidation of any of the urinary metabolites, under any of the conditions tested, except for the marked increase observed for α-TL at room temperature after 1 week in the absence of ascorbate, which may be a rogue result. When stored at room temperature, α-CEHC concentrations tended to decrease with time both in the presence and absence of ascorbate. The largest decrease occurred in the absence of ascorbate by up to approximately 40% after 1 week.

The concentration of α-CMBHC and γ-CEHC showed no consistent and significant change when stored at room temperature either in the presence or absence of ascorbate for up 48 hours, suggesting these metabolites are highly stable. However, after 1 week in the absence of ascorbate α-CMBHC, γ-CEHC and α-TL concentrations were increased.

The concentrations of all the metabolites with the exception of α-CMBHC remained relatively constant over time from 1 week to 6 months, if the samples were stored at -20°C or –80°C. Some variability was, however, observed at the longer storage times for α-CMBHC. This may be explained by the longer retention time of α-CMBHC and relatively low concentrations, resulting in a lower accuracy of measurement.



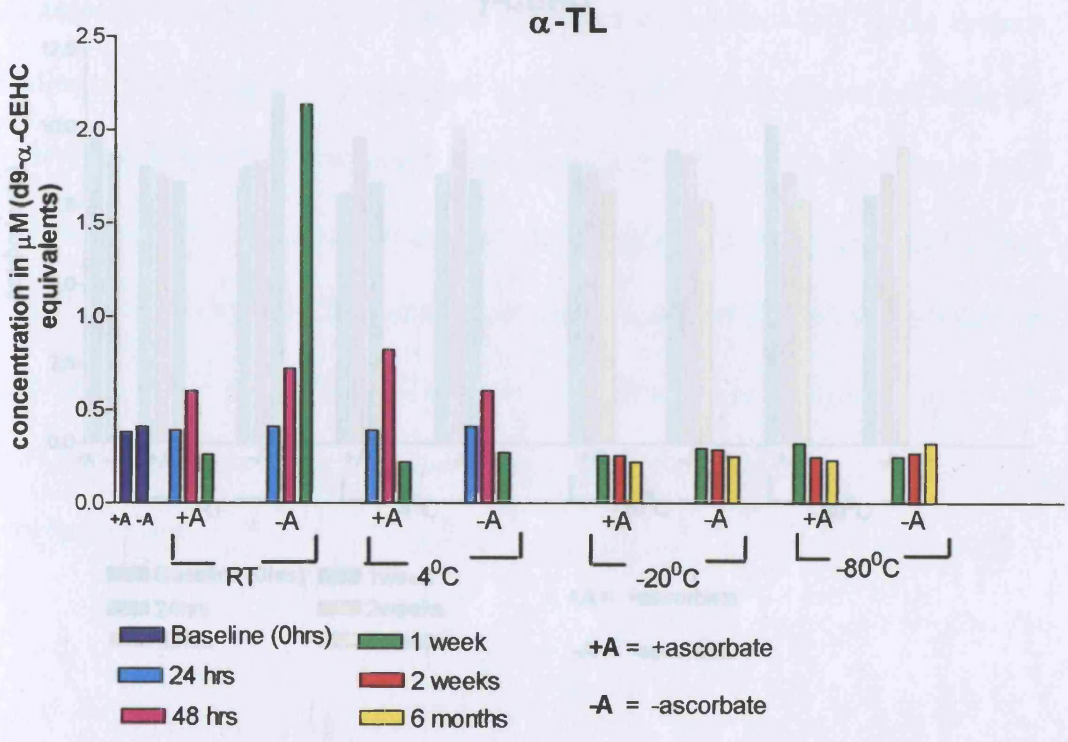


Figure 2.10; The effect of different storage conditions on the level of endogenous urinary metabolites detected, α-CEHC and α-TL (each bar n=1)

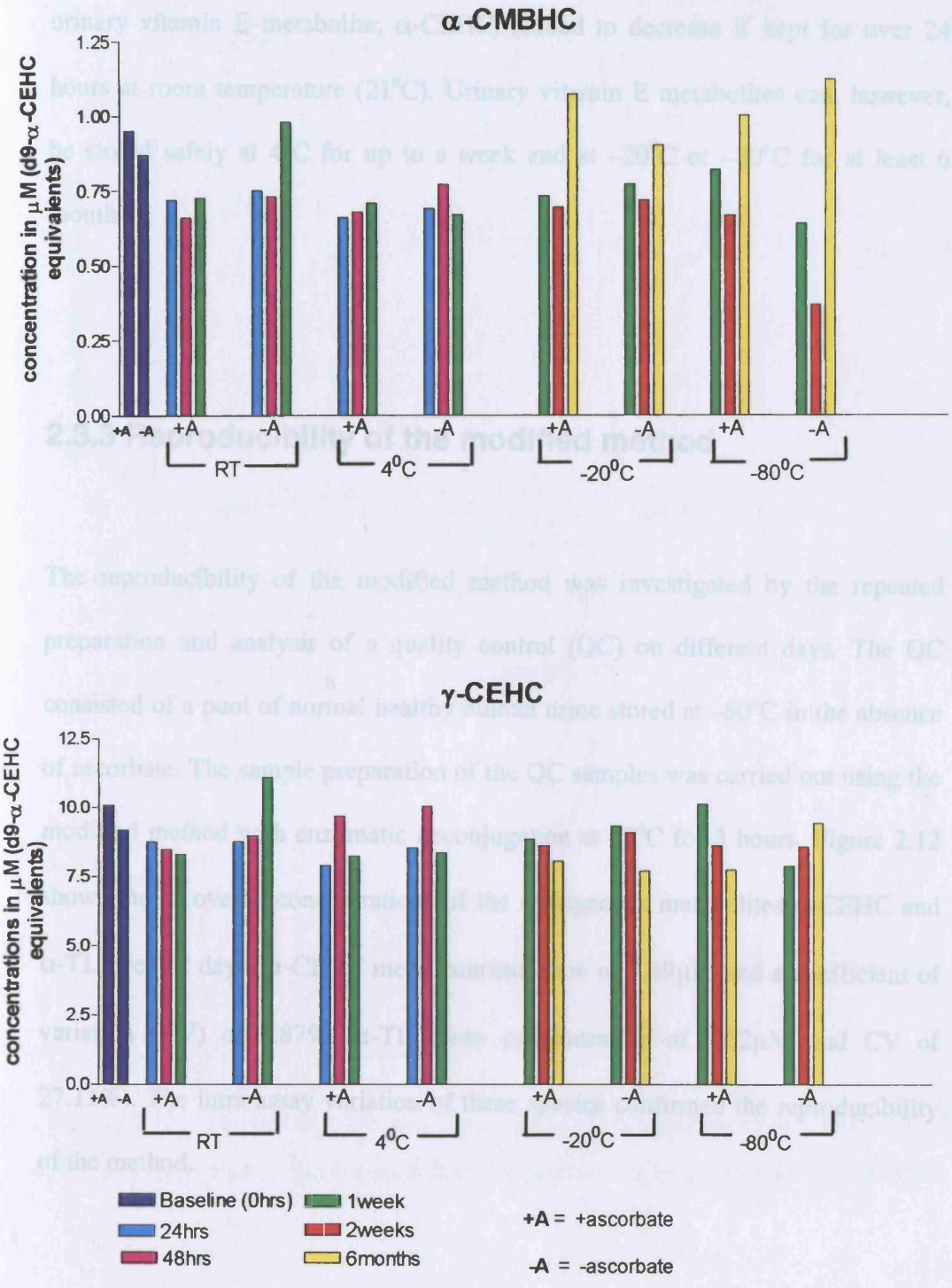


Figure 2.11; The effect of different storage conditions on the level of endogenous urinary metabolites detected, α-CMBHC and γ-CEHC (each bar n=1)

It was concluded that the addition of ascorbate was not necessary and that the urinary vitamin E metabolite, α-CEHC, tended to decrease if kept for over 24 hours at room temperature (21°C). Urinary vitamin E metabolites can, however, be stored safely at 4°C for up to a week and at –20°C or –80°C for at least 6 months.

2.3.3 Reproducibility of the modified method

The reproducibility of the modified method was investigated by the repeated preparation and analysis of a quality control (QC) on different days. The QC consisted of a pool of normal healthy human urine stored at –80°C in the absence of ascorbate. The sample preparation of the QC samples was carried out using the modified method with enzymatic deconjugation at 37°C for 3 hours. Figure 2.12 shows the recovered concentrations of the endogenous metabolites α-CEHC and α-TL over 12 days (α-CEHC mean concentration of 7.49μM and a coefficient of variation (CV) of 3.87%; α-TL mean concentration of 1.52μM and CV of 27.11%). The intra-assay variation of these species confirmed the reproducibility of the method.

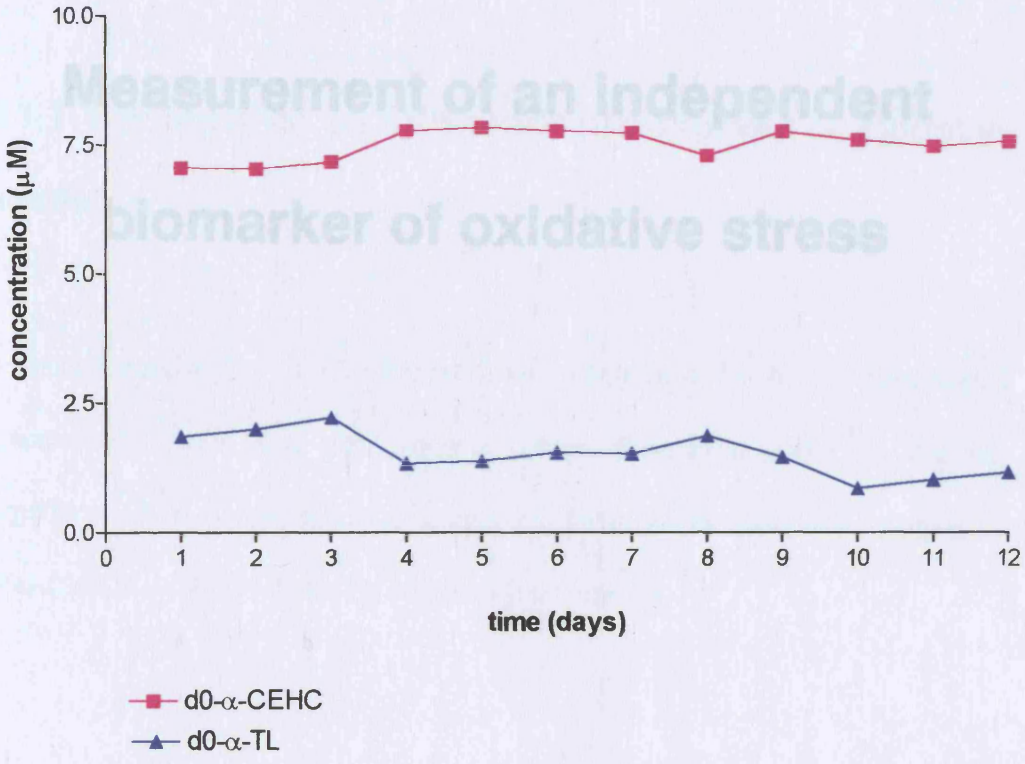


Figure 2.12; The reproducibility of the analysis of endogenous d0-α-CEHC and d0-α-TL over time. Each point for each day represents a single preparation and analysis.

Chapter 3

Measurement of an independent biomarker of oxidative stress

3.1 Independent marker of oxidative stress

3.1.1 The need for an independent biomarker of oxidative stress

α-Tocopheronolactone (α-TL) derived from α-tocopherol has been proposed as a potential biomarker of in vivo oxidative stress (Pope et al 2000). To test this hypothesis, it was necessary to assess an independent and well recognised biomarker of oxidative stress for comparative purposes.

3.1.1.1 Biomarkers of oxidative stress

Oxidative stress is induced by the propagation of free radicals (see chapter 1 section 1.7.1). Free radicals are extremely reactive and short-lived and it is difficult to measure these species and, therefore, oxidative stress directly. Electron spin resonance (ESR) can theoretically be used to detect free radicals. However, in practice it has been found to be necessary to use indirect spin trap methods to detect radical adducts (Poyer et al., 1980). This method is not feasible for application to sample batches in clinical trials. Most methodologies, therefore, measure putative products of oxidative stress. Products of oxidative stress are variable and numerous, as free radicals attack all cell components resulting in

peroxidation of lipids, oxidation of protein and carbohydrates, and oxidative damage to DNA.

Oxidative DNA damage appears to occur continuously in vivo, where reactive oxygen species (ROS) cause lesions to the bases. Damaged DNA is repaired by DNA repair enzymes, which remove modified bases from the DNA. Guanine is the DNA base most prone to oxidative damage resulting in the most predominant lesion, 8-hydroxydeoxyguanosine (8-OHdG). 8-OHdG has been studied widely in both cellular DNA analyses (Kasai et al., 1989) and in non-invasive urinary analysis (Fraga et al., 1990). 8-OHdG and other modified DNA base products need elaborate separation techniques and sensitive detection systems as these products exist in complex matrices in biological samples. In samples such as urine, they are present in the low nano-molar range. Another problem is that these modified DNA base products are difficult to measure due to the complexity of the preparation procedure which results in their artefactual formation and/or modification (Halliwell, 2000; Douki et al., 1996).

Other frequently used markers of oxidative stress are products of lipid peroxidation. A direct approach would involve the measurement of primary products of peroxidation, the hydroperoxides. However, hydroperoxides are unstable, so indirect measures are frequently used which employ determination of secondary or end products derived from further oxidation of the hydroperoxides. The most frequently quoted index of lipid peroxidation is the aldehyde, malondialdehyde (MDA). MDA is a three carbon, low-molecular weight aldehyde that can be produced from free radical attack on polyunsaturated fatty acids. Documented methods to measure MDA either involve the measurement of free

MDA or a MDA derivative. The most common and simple method employs measurement of an MDA derivative, where MDA reacts with thiobarbituric acid (TBA) at low pH and elevated temperature to produce fluorescent and pigmented adducts, referred to as thiobarbituric acid reactive substances (TBARS). This method is however non-specific, when analysing extracts from biological fluids as other low molecular weight aldehydes are also able to react with TBA. Asakawa and Matsushita (1980) demonstrated that MDA levels detected by this method are increased by artefactual lipid peroxidation during sample processing. Direct measurement of free MDA have involved HPLC techniques with spectrophotometric detection (Esterbauer et al., 1984; Tsaknis et al., 1998; Hultqvist et al., 1997). These methods offer higher specificity and sensitivity than the TBARS assays.

Other urinary aldehydes, such as non-polar aldehydes and carbonyl compounds have been used as indicators of in vivo lipid peroxidation. These include butanal, butan-2-one, hexa-2-enal and hexanal. Aldehyde excretion, however, has also proved to be unreliable as their levels are influenced by many factors including energy status, physical activity, environmental temperature and the intake of peroxides in the diet (Draper et al., 2000).

A hydrocarbon breath test measures exhalation of a group of volatile hydrocarbons, the alkanes, formed by in vivo lipid peroxidation of polyunsaturated fatty acids (PUFAs). The alkanes most frequently measured include ethane and pentane. Kivits et al (1981) demonstrated that an increased recovery of exhaled ethane resulted from increased oxidation of ω-3-PUFA, and

oxidation of ω-6-PUFAs was responsible for increased pentane recovery. Practical limitations of this non-invasive approach include the lack of standardised methods of collecting, processing and analysing expired air and the fact that in ambient air the levels of ethane and pentane are greater than in expired air and can easily contaminate the breath samples.

Many studies have measured a group of compounds called the F_2-isoprostanes, a family of eicosanoid like biomolecules, as a sensitive marker of in vivo oxidative stress. The F_2-isoprostanes are lipids that are non-enzymatically derived isomers of the prostaglandins, formed in vivo by free radical mediated oxidation of arachidonic acid. Pratico et al (1998b) have reported the stability of these species in biological specimens. Auto-oxidation can be minimised in lipid containing samples, such as plasma, if immediately snap frozen. Urinary concentrations of isoprostanes did not appear to be affected by auto-oxidation when stored at room temperature for up to seven days.

3.1.1.2 Selecting a biomarker of in vivo oxidative stress

To select a recognised biomarker of in vivo oxidative stress for comparative studies with vitamin E metabolites, it was important to consider its specificity and stability. Isoprostanes (iPs) are chemically very stable compared to other lipid oxidation products which have been used as indices of oxidative stress, such as thiobarbituric acid reactive substances (TBARS) and exhaled alkanes (pentane and ethane). The urinary F_2-isoprostanes provide a sensitive, specific and non-

invasive index of lipid peroxidation. Sensitive and specific analytical techniques described in the literature for the measurement of F_2-isoprostanes, include gas chromatography-mass spectrometry (GC-MS) (Roberts et al., 1996; Lawson et al., 1998; Proudfoot et al., 1999; Bessard et al., 2001) and enzyme immuno-assays (EIA) (Proudfoot et al., 1999; Bessard et al., 2001).

3.1.2 Isoprostanes as indices of oxidative stress

3.1.2.1 History and chemistry of the isoprostanes

The isoprostanes were first documented as in vitro auto-oxidation products of polyunsaturated fatty acids in 1976 (Pryor et al., 1976). It was not, however, until 1990 that studies by Morrow and coworkers, showed that the isoprostanes were produced in vivo in humans (Morrow et al., 1990). The F_2-isoprostanes consist of 4 regioisomers (class III, IV, V, VI) (figure 3.1), which can exist in eight racemic diastereomeric forms (figure 3.2). The most studied of these are the isoprostanes isomeric to $PGF_{2\alpha}$, especially the class III F_2-isoprostane ($iPF_{2\alpha}$-III) species, 8-iso $PGF_{2\alpha}$ (figure 3.2). Studies have shown that this isomer is also produced as a minor product of platelet COX-1 activity in response to stimuli such as collagen, thrombin and arachidonic acid. However, Pratico et al (1998a) found that in practice COX-1 activity did not contribute significantly to the total urinary 8-iso$PGF_{2\alpha}$ concentration. This was shown by the failure of COX-1 inhibition to

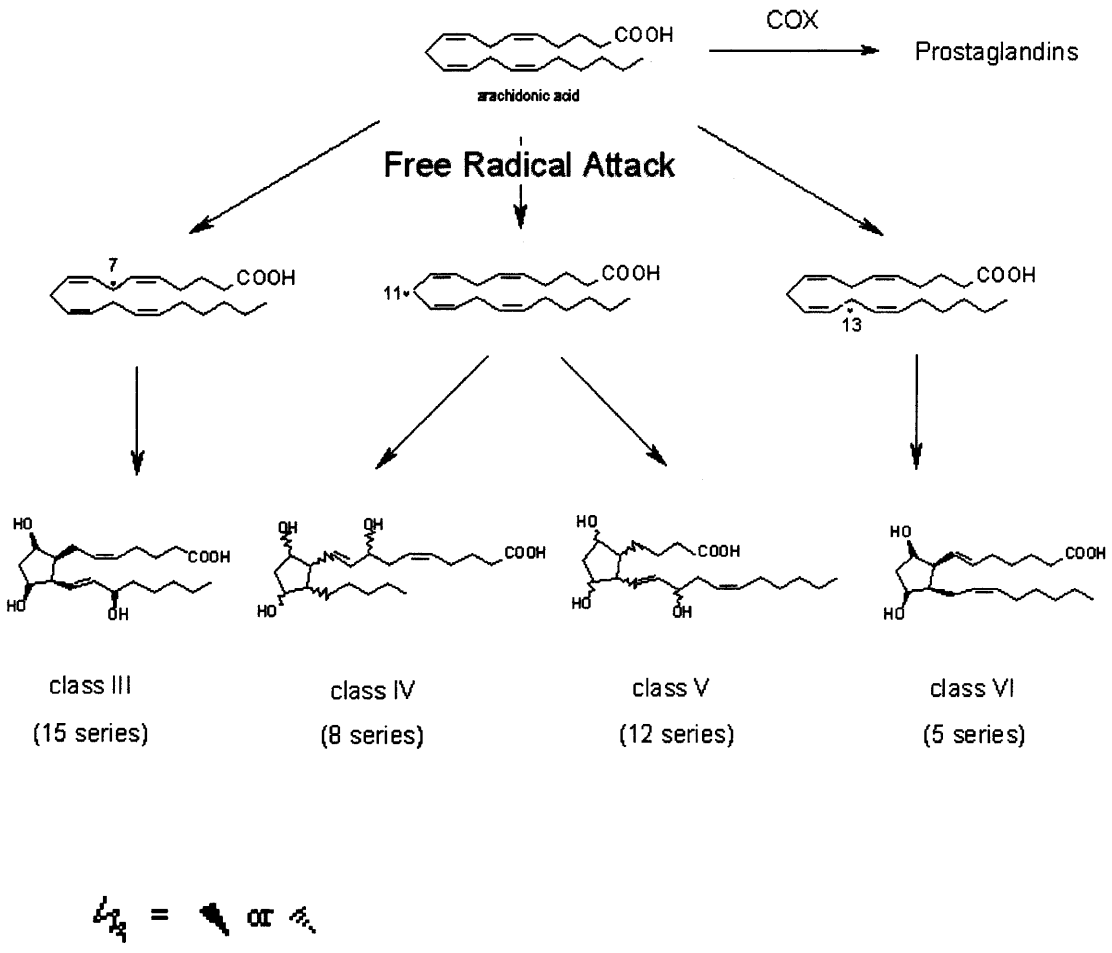
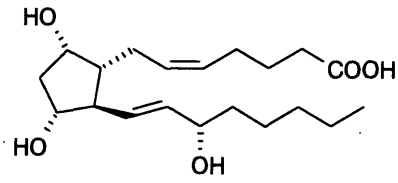
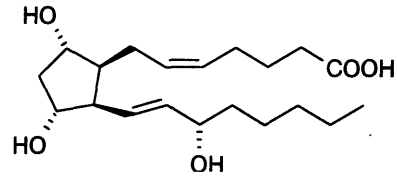


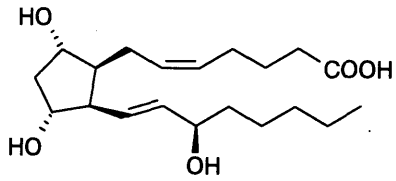
Figure 3.1; Formation of the four regioisomeric classes of F2-isoprostanes from arachidonic acid



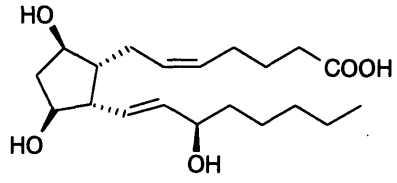
$PGF_{2\alpha}$ (class III)



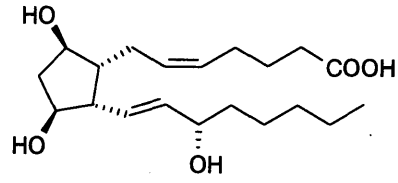
8-iso-$PGF_{2\alpha}$



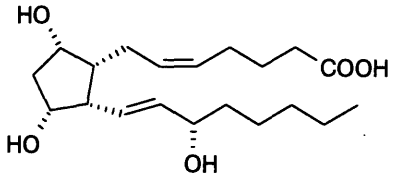
15*R*-8-iso-$PGF_{2\alpha}$



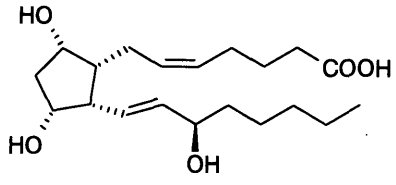
***ent*-8-iso-$PGF_{2\alpha}$**



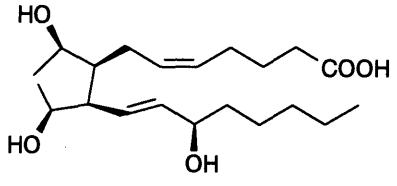
15S-*ent*-8-iso-$PGF_{2\alpha}$



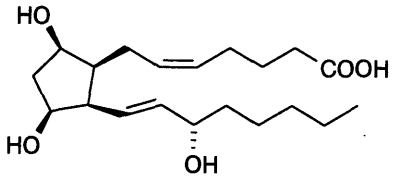
12-iso-$PGF_{2\alpha}$



15*R*-12-iso-$PGF_{2\alpha}$



***ent*-12-iso-$PGF_{2\alpha}$**



15S-*ent*-12-iso-$PGF_{2\alpha}$

Figure 3.2; Structure of prostaglandin $PGF_{2\alpha}$ and the eight racemic diastereomeric forms of class III F_2-isoprostanes

suppress elevated levels of 8-isoPGF$_{2\alpha}$ in urine of individuals under oxidative stress. A number of studies have shown that the F_2-isoprostanes are reliable indicators of lipid peroxidation and oxidative stress (Pratico et al., 1998a;Roberts and Morrow, 2000). Elevated levels have been detected for example in patients with cardiovascular disease (Reilly et al., 1997), Alzheimer's disease (Pratico et al., 1998b; Montine et al., 1999), Down's syndrome (Pratico et al., 2000b) asthma (Dworski et al., 2001; Wood et al., 2000) and both type-1 and type-2 diabetes (Davi et al., 1999; Devaraj et al., 2001a). It was found that the elevated levels of F_2-isoprostanes in type-2 diabetic patients could be significantly reduced by supplementation with α-TH (Devaraj et al., 2001). This finding suggested a possible use of the isoprostanes for monitoring antioxidant therapies.

3.1.2.2 Methods for analysis and measurement of isoprostanes

The two most commonly used methods for measuring urinary F_2-isoprostanes are by GC-MS and enzyme immuno-assay (EIA).

The GC-MS and EIA methods have been compared by two research groups. Proudfoot et al (1999) found that GC-MS gave higher results for 8-isoPGF$_{2\alpha}$ concentration than EIA, which they explained by GC-MS failing to discriminate between a number of different F_2-isoprostane isomers, whereas EIA was specific for a particular isomer. In contrast, Bessard et al. (2001) reported lower

concentrations of 8-isoPGF$_{2\alpha}$ using GC-MS than EIA, and concluded that EIA was less specific.

The fundamental differences between the GC-MS and enzyme-immuno assay (EIA) techniques is that EIA measures F_2-isoprostanes that bind to an antibody raised against 8-isoPGF$_{2\alpha}$, whereas GC-MS measures isomers with common retention times to an internal standard d4-8-isoPGF$_{2\alpha}$. 8-isoPGF$_{2\alpha}$ isomers that may not be present in the GC-MS peak may, therefore, have a high cross-reactivity with the antibody, and isomers that co-elute may not react with the antibody of the EIA.

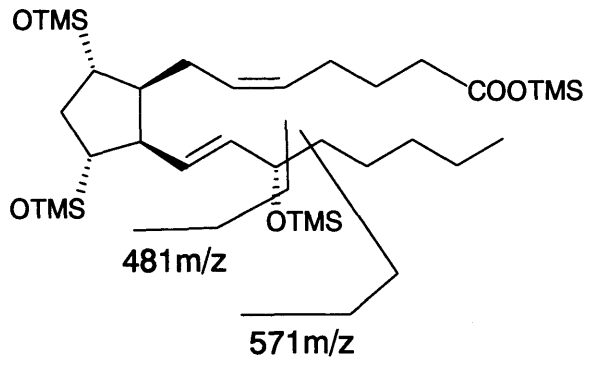
The F_2-isoprostanes are widely considered to be a 'gold standard' for the measurement of in vivo lipid peroxidation as a result of oxidative stress (Pratico, 1999). Urinary measurements of these chemical species provide a non-invasive, and direct approach to measure in vivo oxidative stress, with no in vitro artefactual oxidation. 8-isoPGF$_{2\alpha}$ is the only isomer for which a deuterated standard is commercially available, which is important for quantitation of endogenous levels present in the samples.

The method published by Pratico et al to measure 8-isoPGF$_{2\alpha}$ using GC-MS was refined as described and validated below.

3.2 Method development for the measurement of urinary isoprostanes

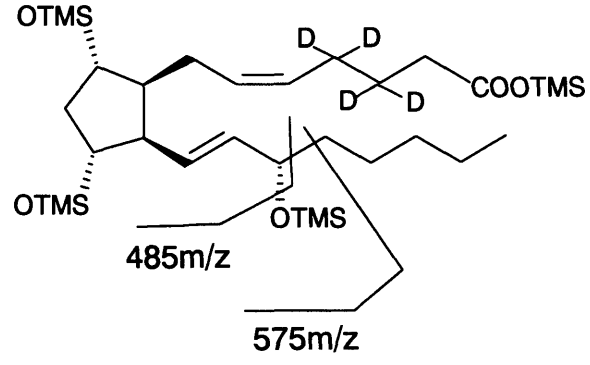
3.2.1 Gas chromatographic-mass spectrometric analysis of 8-iso-$PGF_{2\alpha}$ standards

It was considered necessary to first establish a method that would measure efficiently both deuterated and undeuterated forms of 8-iso-$PGF_{2\alpha}$ standards. The deuterated standard d4-8-iso-$PGF_{2\alpha}$, contains four hydrogen atoms replaced by deuterium atoms (figure 3.3). Hence d4-8-iso-$PGF_{2\alpha}$ is characteristically heavier than its undeuterated form by four mass units enabling the two species to be distinguished using gas chromatography-mass spectrometry (GC-MS). d4-8-iso-$PGF_{2\alpha}$ is therefore a potential internal standard for the quantitation of endogenous 8-iso-$PGF_{2\alpha}$ levels during the analysis of extracted urine samples.



undeuterated, d0-8-isoPGF$_{2\alpha}$

M+ 642



deuterated, d4-8-isoPGF$_{2\alpha}$

M+ 646

Figure 3.3 Principal fragments of trimethyl silyl (TMS) derivatives of undeuterated and deuterated 8-isoPGF$_{2\alpha}$

3.2.1.1 Detection and analysis of deuterated and undeuterated 8-iso-$PGF_{2\alpha}$ standards by GC-MS

Materials

8-isoprostane standards 8-isoprostaglandin $F_{2\alpha}$ (CAY-16350) and 8-isoprostaglandin $F_{2\alpha}$-d4 (CAY-316350) were supplied by *Cayman Chemicals*. These standards were made up into stock solutions of 20ng/ml in methanol and stored at –20°C. In this state they are reported by the manufacturer to be stable for up to six months. The trimethyl-silyl derivatising agents used included N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and BSTFA with 1% trimethyl-chlorosilane (TMCS), and were purchased from *Pierce Chemicals Ltd.* All other chemicals were purchased from *Sigma-Aldrich* unless otherwise mentioned.

Preparation and analysis of standards

Analysis of these standards by GC-MS, was initially carried out by modifying the method of Bessard et al (2001). The standards were derivatised to their trimethyl-silyl derivatives using 60µl BSTFA (+1%TMCS). Bessard's method involved no incubation for the derivatisation, suggesting immediate derivatisation. Derivatisation conditions were adapted in the present study and incubated at 45°C for 20 minutes to ensure derivatisation reached completion. GC-MS analysis of the derivatised standards was performed in electron impact (EI) mode at 70

electron volts, using a Hewlett Packard GC-MS system (HP-5973 mass selective detector, HP-6890 series GC instrument with an automatic auto sampler, and HP-Chem station for data acquisition). The initial oven temperature of 130°C was maintained for 1 minute, then increased to 300°C at 18°C/min, which was then held for 2 minutes. The oven temperature was then increased to 310°C at 4°C/min and maintained at this temperature for 5 minutes to purge the column. The total run time was 19.94 minutes. The injector system was run in splitless mode. The helium carrier gas was kept at a constant flow rate of 1ml/min. GC-MS temperatures were set as follows; injector at 250°C, interface at 300°C, source at 220°C and the quadrupole at 100°C.

The mass spectrometer (MS) was programmed to run the above method in both complete scan mode and in selected ion-monitoring (SIM) mode. For the selected ion-monitoring mode the principal fragments were selected for identification and are shown in figure 3.4. The fragments of d0-8-isoPGF$_{2\alpha}$ include 571m/z ion (loss of C_5H_{11}) and 481m/z (loss of C_5H_{11} and trimethyl-silyl (TMSO)). The equivalent corresponding fragments of d4-8-isoPGF$_{2\alpha}$ were 575m/z and 485m/z. Quantification was achieved by measuring the peak heights of the highest abundance mass ions for both undeuterated and deuterated 8-isoPGF$_{2\alpha}$, which were the ions 481m/z and 485m/z respectively in SIM mode.

The chromatographic peaks for both undeuterated and deuterated 8-isoPGF$_{2\alpha}$ standards were successfully identified according to their characteristic fragmentation demonstrated by the corresponding mass spectra for the peaks (figure 3.4 and 3.5). When, however, standards were analysed at a concentration

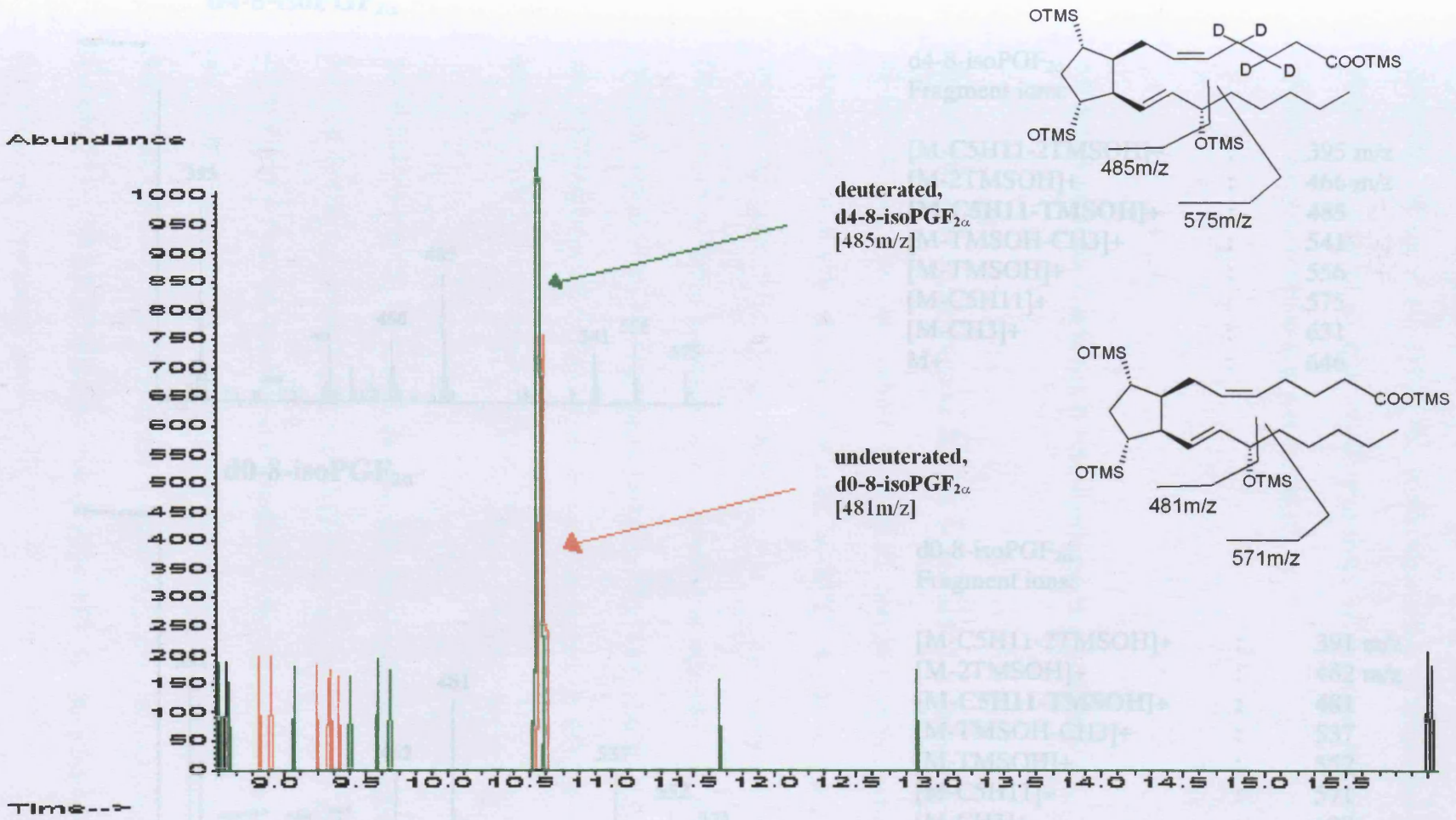
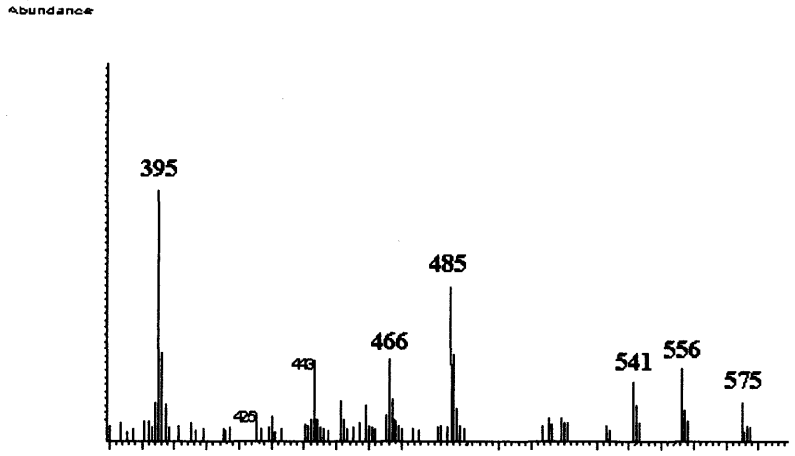


Figure 3.4; Extracted ion chromatogram of d0- and d4- 8-isoPGF$_{2\alpha}$

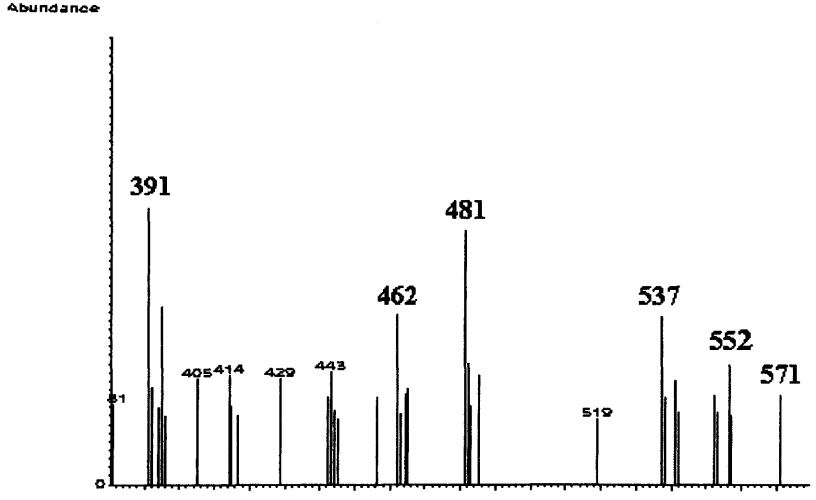
d4-8-isoPGF$_{2\alpha}$



d4-8-isoPGF$_{2\alpha}$
Fragment ions:

[M-C5H11-2TMSOH]+	:	395 m/z
[M-2TMSOH]+	:	466 m/z
[M-C5H11-TMSOH]+	**:**	**485**
[M-TMSOH-CH3]+	:	541
[M-TMSOH]+	:	556
[M-C5H11]+	:	575
[M-CH3]+	:	631
M+	:	646

d0-8-isoPGF$_{2\alpha}$



d0-8-isoPGF$_{2\alpha}$
Fragment ions:

[M-C5H11-2TMSOH]+	:	391 m/z
[M-2TMSOH]+	:	462 m/z
[M-C5H11-TMSOH]+	**:**	**481**
[M-TMSOH-CH3]+	:	537
[M-TMSOH]+	:	552
[M-C5H11]+	:	571
[M-CH3]+	:	627
M+	:	642

Figure 3.5; Characteristic fragment ions of d4- and d0-8-isoPGF$_{2\alpha}$.

equivalent to that which might be expected in human urine i.e. 88-442pg/mg of creatinine (Wang et al., 1995), the peaks were either undetectable or of poor shape. In an attempt to improve the sensitivity of detection of the isoprostane species, the derivatisation step was modified.

Modification of the conditions for the derivatisation of 8-isoPGF$_{2\alpha}$

Initially the derivatising solution, BSTFA (+1%TMCS), described by Bessard et al (2001) was used. In contrast to Bessard, however, derivatisation in this experiment was achieved by incubation at 45°C for 20 minutes. Different derivatising agent mixes were compared over a time course of incubation. Deuterated 8-isoPGF$_{2\alpha}$ standard, at a concentration equivalent to 600pg/ml if spiked in 4ml of urine, was derivatised using BSTFA (+1% TMCS), BSTFA alone and BSTFA/acetonitrile (1:1). Samples were incubated at 45°C at 10 minute intervals from 20 to 100 minutes (figure 3.6).

BSTFA (+1% TMCS) yielded no defined chromatographic peaks at the level of standard derivatised. It was found that BSTFA/acetonitrile gave the best recovery of the silylated standard, showing approximately a five fold greater recovery than that of BSTFA alone (figure 3.6). There was no consistent change in recovery after 20 minutes and it was decided that in all further studies, derivatisation should be carried out using BSTFA/acetonitrile (1:1), at 45°C for 80 minutes to ensure derivatisation went to completion.

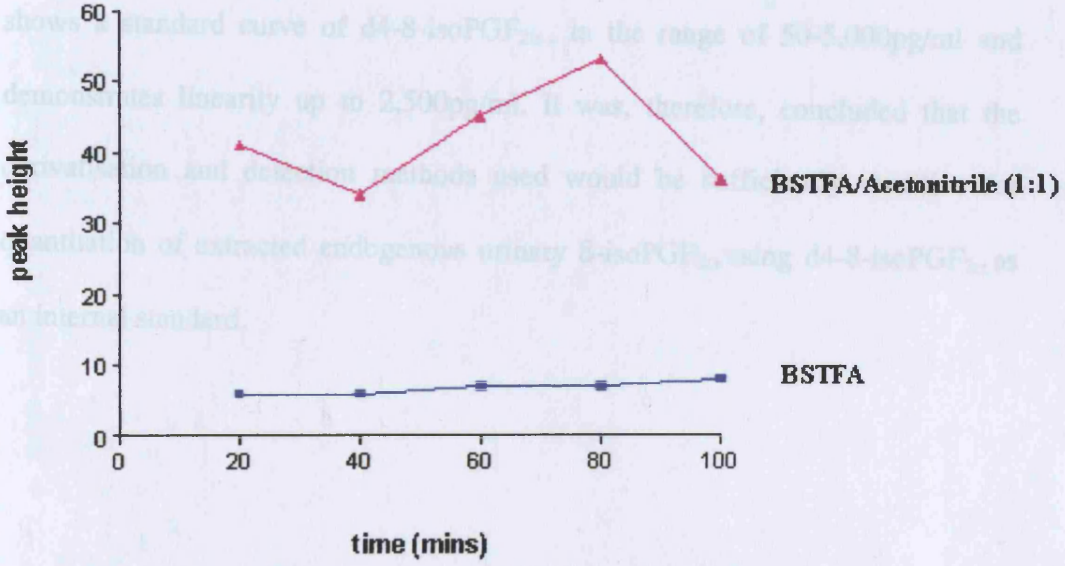


Figure 3.6; The effects of different derivatising agents and incubation times on recovery of d4-8-isoPGF$_{2\alpha}$

Range and proportionality of detection of 8-isoPGF$_{2\alpha}$

A series of d4-8-isoPGF$_{2\alpha}$ concentrations were prepared (equivalent to the concentrations expected in urine samples) in order to estimate the concentration range within which effective and reliable detection is obtained using the derivatising conditions and GC-MS program set out in the protocol. Figure 3.7 shows a standard curve of d4-8-isoPGF$_{2\alpha}$, in the range of 50-5,000pg/ml and demonstrates linearity up to 2,500pg/ml. It was, therefore, concluded that the derivatisation and detection methods used would be sufficiently sensitive for quantitation of extracted endogenous urinary 8-isoPGF$_{2\alpha}$ using d4-8-isoPGF$_{2\alpha}$ as an internal standard.

3.2.2 Method development for the extraction of 8-isoPGF$_{2\alpha}$ from urine

3.2.2.1 Polarity dependent solid phase extraction

Bessard's (2001) method of extraction is relatively simple, involving two solid phase extraction (SPE) steps and relies on the characteristic polarity of the isoprostanes for their extraction.

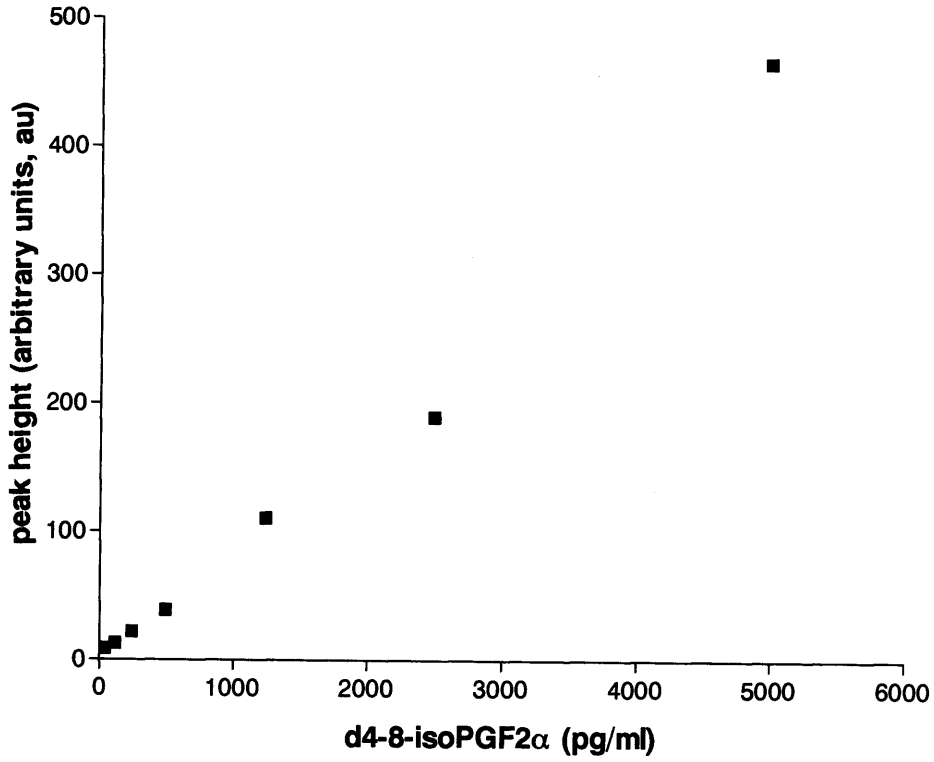


Figure 3.7; Standard curve of d4-8-iso-$PGF_{2\alpha}$ in range 50-5,000pg/ml.

Materials

C18 10ml cartridges (100mg sorbent mass) and NH_2 10ml cartridges (100mg sorbent mass) were supplied by Isolute.

Extraction protocol using solid phase extraction cartridges

The method involved using 4ml of urine (a morning void of fresh urine collected from a 26 year old female colleague (no vitamin supplements consumed for at least 2 years, non-smoker, teetotal)) spiked with deuterated (d4-) and undeuterated (d0-) 8-isoPGF$_{2\alpha}$ standards at concentrations equivalent to 2,000pg/ml and in the range of 100-3,200pg/ml respectively. The first SPE step was carried out using a C18 cartridge, with a solvent program as follows;

1) cartridges primed with 2ml methanol and 2ml dH_2O (pH2.5)
2) sample loaded onto cartridge
3) washed with 6ml dH_2O (pH2.5), and 6ml acetonitrile/water (15:85)
4) eluted using 4ml hexane/ethyl-acetate/propan-2-ol (30:65:5).

The eluate was then further extracted using an NH_2 cartridge. The solvent program for the NH_2 cartridge was as follows;

1) cartridges primed with 5ml hexane
2) sample loaded onto cartridge
3) washed with 5ml hexane/ethylacetate (30:70), and 5ml acetonitrile
4) eluted using ethylacetate/methanol/acetic acid (10:85:5).

The extract was evaporated to dryness under a gentle stream of N_2 and then derivatised as described in the modified method in section 3.2.1.1, using BSTFA/acetonitrile at 45°C for 80 minutes. The derivatised extract was analysed by GC-MS in both scan and SIM modes as detailed for standards in section 3.2.1.1.

Results

The chromatograms of the extracted samples showed evidence of peaks for do- and d4-8-isoPGF$_{2\alpha}$ at their characteristic retention times identified for the standards in section 3.2.1.1. Figure 3.8 shows the extracted ion chromatogram for d0-8-isoPGF$_{2\alpha}$ (481m/z) and d4-8-isoPGF$_{2\alpha}$ (485m/z), and the respective mass spectra for the two species. Although the peaks for the two isoprostane forms can be identified, the signal to noise ratio is low, and the respective mass spectra for the 8-isoprostane peaks contained many other fragment ions that could not be explained by these specific isoprostane species alone. This suggested that other species may be co-eluting with the isoprostane species of interest. It was subsequently concluded that this extraction procedure was not sufficiently specific to clean the sample adequately, and allow clear detection of the 8-isoPGF$_{2\alpha}$ species.

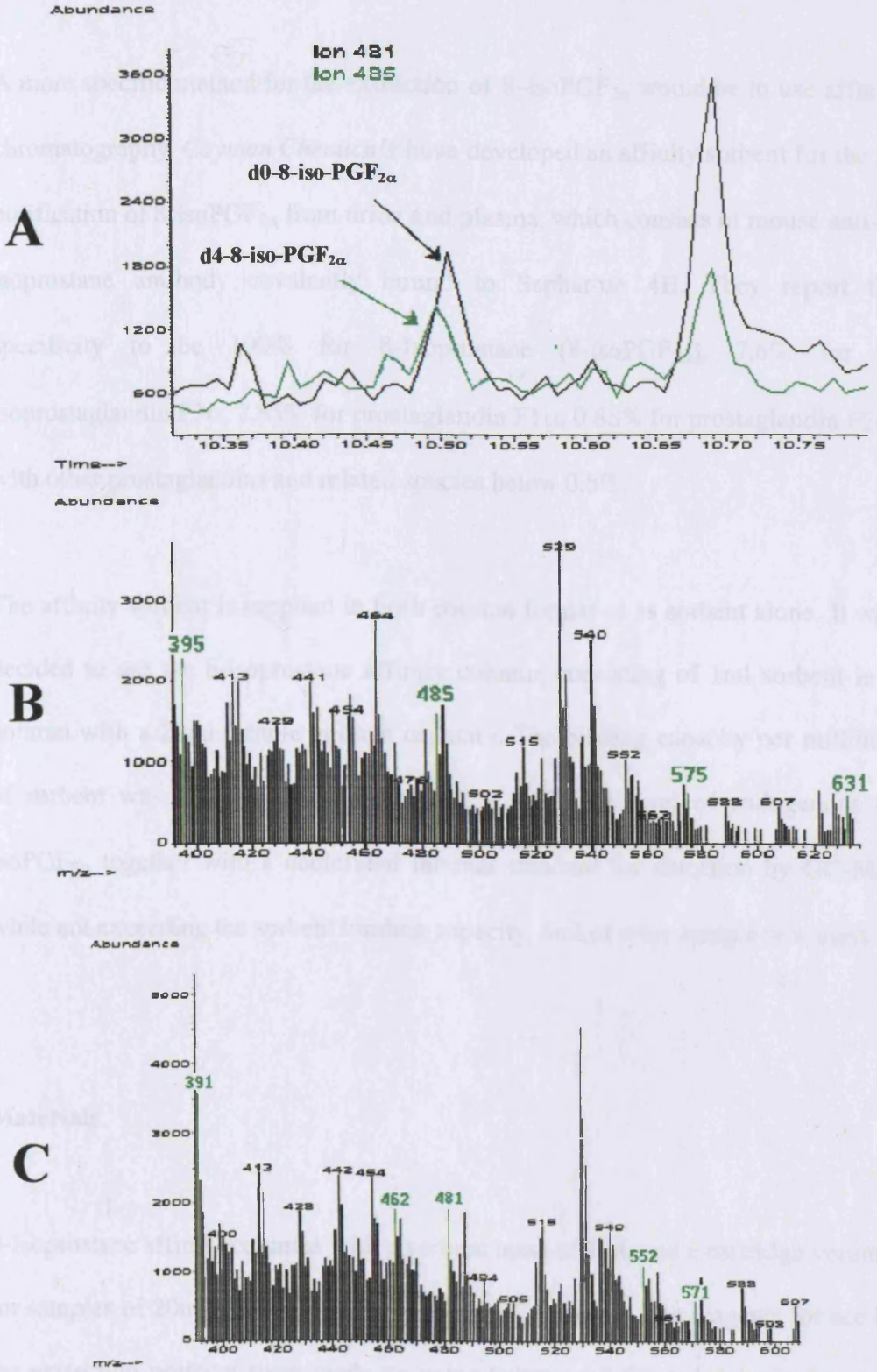


Figure 3.8; Extracted ion chromatogram of ions 485m/z and 481m/z for extracted urine spiked with 2000pg/ml d4 standard and 2000pg/ml d0 standard (A), and mass spectra for peaks representing d4 (B) and d0-8-isoPGF$_{2\alpha}$ (C).

3.2.2.2 Extraction of 8-isoPGF$_{2\alpha}$ from urine using affinity chromatography

A more specific method for the extraction of 8–isoPGF$_{2\alpha}$ would be to use affinity chromatography. *Cayman Chemicals* have developed an affinity sorbent for the purification of 8-isoPGF$_{2\alpha}$ from urine and plasma, which consists of mouse anti-8-isoprostane antibody covalently bound to Sepharose 4B. They report the specificity to be 100% for 8-Isoprostane (8-isoPGF$_{2\alpha}$), 7.6% for 8-isoprostaglandin F3α, 2.85% for prostaglandin F1α, 0.88% for prostaglandin F2α, with other prostaglandins and related species below 0.5%.

The affinity sorbent is supplied in both column format or as sorbent alone. It was decided to use the 8-isoprostane affinity column, consisting of 1ml sorbent in a column with a 20ml sample volume capacity. The binding capacity per millilitre of sorbent was 10ng. In order to extract a sufficient level of endogenous 8-isoPGF$_{2\alpha}$ together with a deuterated internal standard for detection by GC-MS, while not exceeding the sorbent binding capacity, 4ml of urine sample was used.

Materials

8-Isoprostane affinity columns with a sorbent mass of 1ml, and a cartridge volume for samples of 20ml were supplied by *Cayman Chemicals*. The reagents for use in the extraction protocol were made up using "ultrapure" filtered deionised water.

All chemicals were purchased from *Sigma-Aldrich Chemicals* unless otherwise stated. The reagents include;

i) eicosanoid affinity column buffer ; 0.1M phosphate buffer: 13.3g K_2HPO_4, 3.22g KH_2PO_4, 0.5g NaN_3 and 29.2g NaCl made to 1litre with "ultrapure" water, pH7.4

ii) eicosanoid affinity column elution solution ; 95% ethanol in 5% "ultrapure" water

iii) "ultrapure" water

Derivatisation materials were as used in section 3.2.1

Extraction protocol and analysis method using affinity columns

The extraction procedure was carried out as follows;

1- urine samples were centrifuged at 3,000g for 5 minutes to remove particulate matter

2- 4 ml urine was spiked with undeuterated 8-isoPGF$_{2\alpha}$ standard (to give a final concentration of 500pg/ml), and mixed by inverting

3- affinity column extraction

   i) storage column buffer was allowed to drain out

   ii) urine sample loaded

   iii) column washed with 10ml column buffer, followed by 10ml "ultrapure" water

   iv) water allowed to completely drain from the column

   v) bound isoprostane eluted using 5ml elution solution, and collected

vi) columns recycled by washing with 10ml "ultrapure" water, followed by 5ml column buffer and allowed to drain before storing at 4°C in 5ml column buffer

4- eluate evaporated to dryness under gentle stream of N_2

5- derivatised using 60µl BSTFA/acetonitrile (1:1) at 45°C for 80 minutes

6- analysed on GC-MS, using method described in section 3.2.1.1.

Results

The extraction of urine samples using the affinity column resulted in chromatograms with significantly lower baseline noise and a larger sharper peak for the 8-iso-$PGF_{2\alpha}$ (figure 3.9) compared with the solid phase extraction method described in section 3.2.2.1 (figure 3.8). The mass spectra for the peaks of interest contain the fragments relevant to the 8-iso$PGF_{2\alpha}$ species with few other fragments. The differences observed following the two extraction methods suggested that the affinity column method was much more efficient at cleaning the samples resulting in easier detection and therefore more reliable quantification of the species of interest.

The 8-iso$PGF_{2\alpha}$ peak, with a retention time of 10.5 minutes, was identified using the scan acquisition mode and the selected ion-monitoring (SIM) mode in an unspiked urine sample and a sample spiked with 500pg/ml d0-8-iso$PGF_{2\alpha}$ standard. This showed that endogenous levels were detectable using this

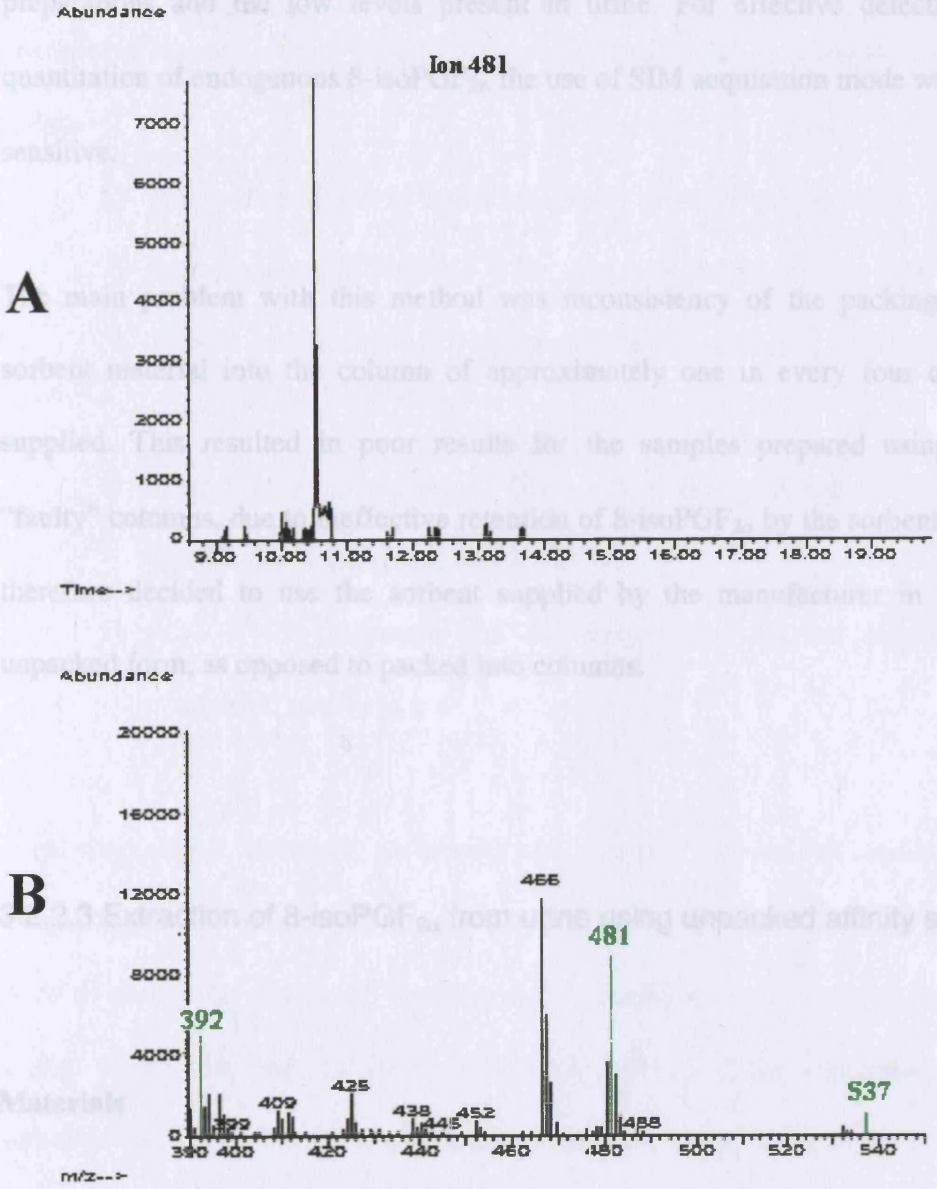


Figure 3.9; Extracted ion chromatogram (A) and mass spectrum (B) for d0-8-isoPGF$_{2\alpha}$ extracted from urine, spiked with 500pg/ml d0-8-isoPGF$_{2\alpha}$ using affinity chromatography columns and SCAN analysis

extraction and analysis method. Endogenous levels, however, were not always detectable in the scan mode, possibly due to variation between sample preparations and the low levels present in urine. For effective detection and quantitation of endogenous 8-isoPGF$_{2\alpha}$ the use of SIM acquisition mode was more sensitive.

The main problem with this method was inconsistency of the packing of the sorbent material into the column of approximately one in every four columns supplied. This resulted in poor results for the samples prepared using these "faulty" columns, due to ineffective retention of 8-isoPGF$_{2\alpha}$ by the sorbent. It was therefore decided to use the sorbent supplied by the manufacturer in its free unpacked form, as opposed to packed into columns.

3.2.2.3 Extraction of 8-isoPGF$_{2\alpha}$ from urine using unpacked affinity sorbent

Materials

8-Isoprostane affinity sorbent was supplied by *Cayman Chemicals* in 1ml aliqouts. Reagents used were the same as described for the affinity column technique.

Extraction protocol using the sorbent "batch" procedure

The batch procedure for the extraction of 8-isoPGF$_{2\alpha}$ from urine described by the manufacturer was initially used and involved a series of centrifugation steps as follows;

1) 1ml sorbent added to 1ml urine, gently rotated for 30-60mins
2) centrifuged at 1,500g for 5 mins, then supernatant pippetted off
3) sorbent washed with 1ml column buffer, centrifuged, then supernatant removed
4) sorbent pellet resuspended in 0.5ml elution solution (95% EtOH), centrifuged, supernatant collected
5) step 4 was repeated, the two extractions pooled together and evaporated to dryness.

It was necessary, however, to modify the original protocol to obtain cleaner chromatograms. It was found that the washing sequence had to be more thorough prior to elution, to remove all the phosphate remaining from the 0.1M phosphate buffer which hindered the derivatisation step. Also, if the supernatants were removed from too close to the sedimented sorbent layer, some sepharose beads were removed and this caused contamination of the eluate. It was, therefore, necessary to leave a small volume of supernatant above the sorbent mass layer. The final procedure which included the above mentioned adjustments was as follows;

1- Preparation of urine samples

i) urine was centrifuged to remove particulate matter

ii) 4ml urine was aliquoted into 15ml plastic Falcon conical base centrifuge tubes

iii) urine aliquots were spiked with 10µl of 200pg/ml 8-iso-$PGF_{2\alpha}$ standard stock and then vortexed for 30secs

2- Preparation of affinity sorbent

i) sorbent was resuspended in 2ml column buffer, by gently pipetting and expelling to make homogenous, and then aliquoted into the urine sample

ii) mixed by inverting, and then mixed by rotating for 90mins

3- Extraction of 8-isoprostane from urine sample

i) sorbent-urine mixture was centrifuged at 2,000g for 5mins, to sediment the sorbent

ii) supernatant was removed (supernatant devoid of 8-iso$PGF_{2\alpha}$)

iii) sorbent was washed with 5ml column buffer (0.1M phosphate buffer) and vortexed for 30secs

iv) centrifuged at 2,000g for 5mins to sediment sorbent

v) supernatant removed, and steps iii) to v) were repeated (supernatant devoid of 8-iso$PGF_{2\alpha}$)

vi) sorbent washed with 5ml ultrapure dH_2O and vortexed for 30secs

vii) centrifuged at 2,000g to sediment sorbent

viii) supernatant removed, and steps vi) to viii) were repeated twice (supernatant devoid of 8-iso$PGF_{2\alpha}$)

ix) sorbent pellet resuspended in 2.5ml of elution solution (95% EtOH) and vortexed for 30secs

x) centrifuged at 2,000g for 5mins, to sediment sorbent

xi) EtOH phase removed carefully (so as not to disturb sorbent) and collected into 10ml glass tube

xii) steps ix) to xii) were repeated twice

4- Derivatisation of eluate

i) EtOH phase was evaporated to dryness (when approximately 1ml remained this was transferred to a 2ml derivatising vial before completing evaporation)

ii) derivatising agent added (60µl Acentonitrile/BSTFA (1:1)) and incubated at 45^0C for 80 mins

5- Run sequence on GC-MS, as described in 3.2.1.1 in both scan and SIM acquisition modes

6- Regeneration of sorbent

i) sorbent washed with 5ml "ultrapure" dH_2O, this was repeated twice and the washes were discarded

ii) washed with 5ml column buffer (0.1M phosphate buffer), wash discarded

iii) 2.5ml column buffer (0.1M phosphate buffer) added

iv) centrifuged at 2,000g for 5mins, and supernatant removed until a total volume including sorbent mass of 2ml remained

v) sorbents were stored at 4°C

The manufacturer of the sorbent recommended a maximum of five regeneration cycles per sorbent, as each regeneration cycle decreases the resin binding capacity. It was found, however, in the present study that sorbents were reliable after only one regeneration cycle. After the second use of the sorbents a general decrease in extraction efficiency was evident (figure 3.10).

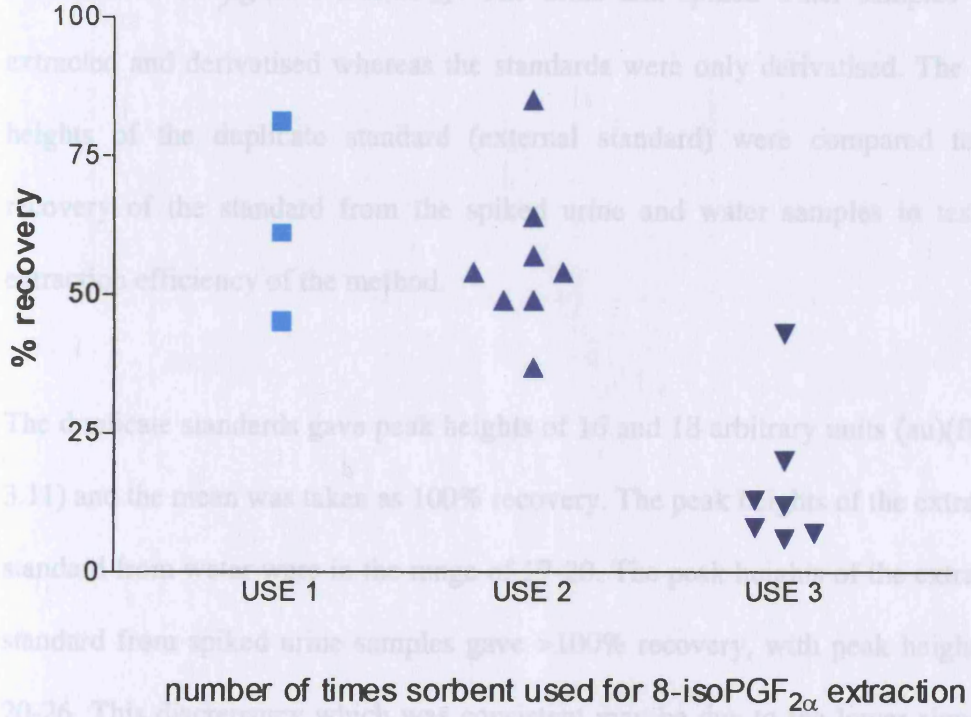


Figure 3.10; Percentage recovery of spiked 8-isoPGF$_{2\alpha}$ from urine after repeated use of the sorbents, compared to an external standard.

Results

In order to test the effectiveness of the method, peak heights of 8-isoPGF$_{2\alpha}$ were compared from triplicate samples of urine and water both spiked with a final concentration of 500pg/ml of d4-8-isoPGF$_{2\alpha}$, unspiked urine and duplicate standards of 500pg/ml 8-isoPGF$_{2\alpha}$. The urine and spiked water samples were extracted and derivatised whereas the standards were only derivatised. The peak heights of the duplicate standard (external standard) were compared to the recovery of the standard from the spiked urine and water samples to test the extraction efficiency of the method.

The duplicate standards gave peak heights of 16 and 18 arbitrary units (au)(figure 3.11) and the mean was taken as 100% recovery. The peak heights of the extracted standard from water were in the range of 17-20. The peak heights of the extracted standard from spiked urine samples gave >100% recovery, with peak heights of 20-26. This discrepancy which was consistent may be due to the lower signal to noise ratio in extracted urine samples. The peak heights of the endogenous d0-8-isoPGF$_{2\alpha}$ in the unspiked urine samples gave a mean concentration of 270 pg/ml.

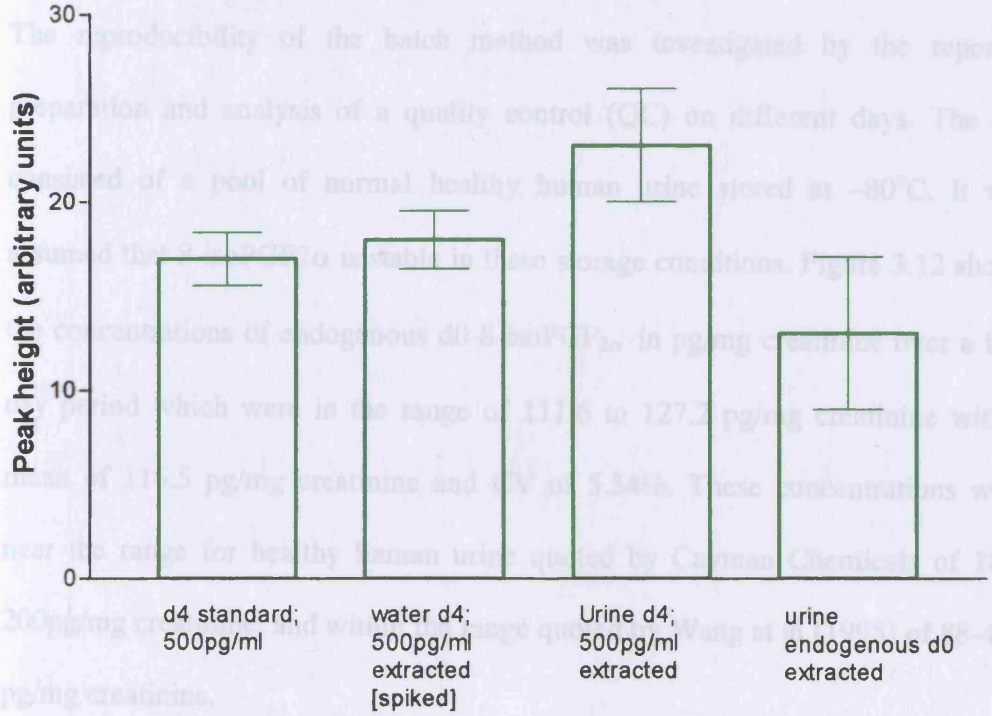


Figure 3.11; The mean peak heights for the d4-8-isoPGF2α standard, the recovered d4-8-isoPGF$_{2\alpha}$ standard from spiked water and urine, and endogenous d0-8-isoPGF$_{2\alpha}$ from urine. NOTE: n=2 for d4 standards and for each remaining column n=3, and error bars represent standard deviation.

3.2.2.4 Reproducibility of the method

The reproducibility of the batch method was investigated by the repeated preparation and analysis of a quality control (QC) on different days. The QC consisted of a pool of normal healthy human urine stored at –80°C. It was assumed that 8-isoPGF2α is stable in these storage conditions. Figure 3.12 shows the concentrations of endogenous d0-8-isoPGF$_{2\alpha}$ in pg/mg creatinine over a five day period which were in the range of 111.6 to 127.2 pg/mg creatinine with a mean of 116.5 pg/mg creatinine and CV of 5.54%. These concentrations were near the range for healthy human urine quoted by Cayman Chemicals of 180-200pg/mg creatinine, and within the range quoted by Wang et al (1995) of 88-442 pg/mg creatinine.

The method was considered sufficiently reproducible to be used to measure urinary 8-isoPGF$_{2\alpha}$ as an independent biomarker of in vivo oxidative stress in samples collected from subjects undergoing an exercise regimen (chapter 4).

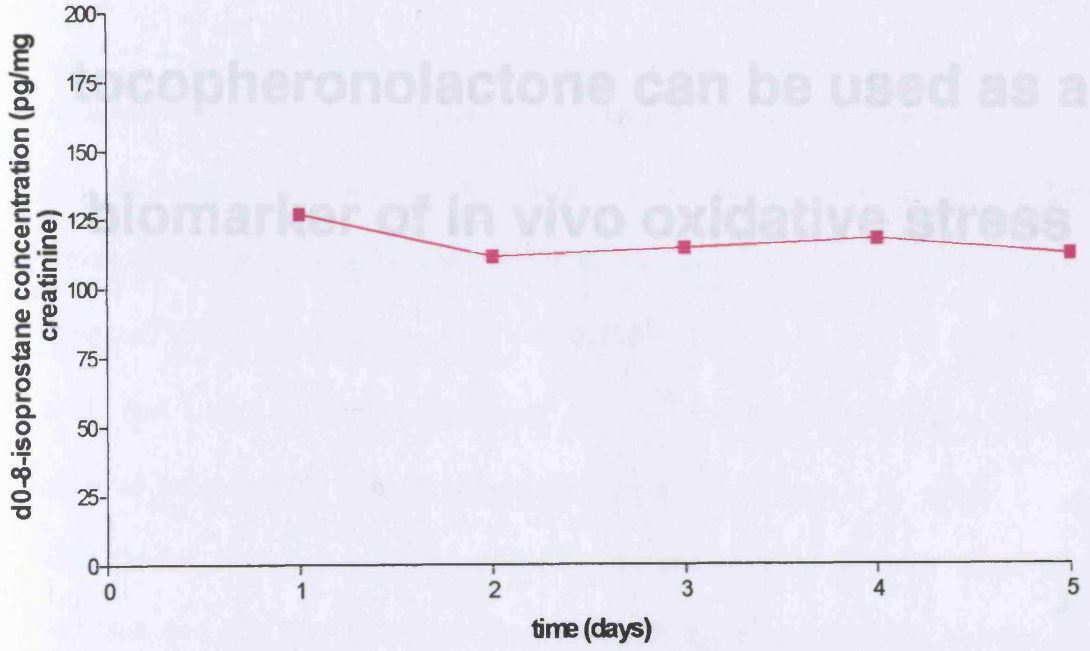


Figure 3.12; The reproducibility of the analysis for endogenous d0-8-isoPGF$_{2\alpha}$ in normal human urine. Each point for each day represents a single preparation and analysis.

Chapter 4

To investigate whether α-tocopheronolactone can be used as a biomarker of in vivo oxidative stress

4.1 In vivo oxidative stress

4.1.1 Investigation of α-tocopheronolactone as a potential biomarker of oxidative stress

As mentioned in chapter 2, α-tocopheronolactone (α-TL) can be detected in urine but it is not entirely clear whether the levels detected are due to artefactual oxidation during sample preparation or whether a proportion is also produced in vivo as a result of natural biological oxidation of α-tocopherol (α-TH). If α-TL is a real metabolite of α-TH and a product of the antioxidant activity of α-TH, its production might be expected to be elevated in states of oxidative stress and excreted in the urine.

This chapter describes a study carried out in collaboration with Dr Mark Goss-Sampson and Ms Kim Hastings, The Centre for Sport and Exercise Science, The University of Greenwich, to examine the effect of endurance exercise on the urinary excretion of α-TL. The project was carried out in accordance with the University of Greenwich Ethics Committee guidelines following both verbal and written consent from each subject involved in the study. Firstly the importance of oxidative stress in medicine and in biological processes of living organisms will be discussed.

4.1.2 Oxidative stress and antioxidant defence

Oxidative stress results from an imbalance of prooxidant and antioxidant activities, where the former outweighs the antioxidant defences (for more detail see chapter 1, section 1.7.1.1).

4.1.2.1 Human diseases and conditions associated with oxidative stress

Oxidative stress has been implicated in the pathogenesis of many disease states and conditions, including atherosclerosis (Ross, 1999), cystic fibrosis (Winklhofer-Roob, 1994; Benabdeslam et al., 1999), diabetes (Dandona et al., 1996; Davi et al., 1999), Parkinson's disease (Beal, 1998), Alzheimer's disease (Mattson, 1995), Down's syndrome (Pastor et al., 1998; Jovanovic et al., 1998), and is also associated with viral infections such as human immuno-deficiency virus (HIV) (Droge et al., 1994), some cancers (Boyd and McGuire, 1991), with aging (Harman, 1993), cigarette smoking (Obata et al., 2000) and exercise which is discussed in more detail below. Oxidative stress may be the cause or a consequence of these conditions (Halliwell and Gutteridge, 1999).

4.1.2.2 Exercise and oxidative stress

The principal factor considered to be responsible for oxidative stress damage during exercise is an increased demand for oxygen supply and consumption to cope with the body's needs for energy. Upon exercising from a state of rest, oxygen uptake by the

human body increases by approximately 10 to 15 fold, and uptake of oxygen by active skeletal muscle can increase by up to 100 fold. Oxidative stress induced by exercise has been postulated to occur through a number of mechanisms.

An increased oxygen flux through the muscle cell mitochondria would increase the release of reactive oxygen species (ROS) resulting in an increase in oxidative stress. The electron transport chain of the mitochondrial inner membrane is well known to be a major source of superoxide ($^{\cdot}O_2^-$) production, resulting in hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH^-$) formation. The electron carriers, NADH-ubiquinone reductase (complex 1) and ubiquinone-cytochrome-c reductase (complex 3) of the electron transport chain are known sites of $^{\cdot}O_2^-$ and H_2O_2 generation via side-reactions with molecular oxygen (Chance et al., 1979).

ROS generation could occur as a result of inflammation and muscle damage, and by the recruitment of macrophages to repair damaged tissues. It was first demonstrated by Davies et al (1982) that muscle damage resulted from severe forced exercise in rats, illustrated by decreased control of mitochondrial respiration and loss of structural integrity of the sarcoplasmic reticulum. Exercise had also been shown to be asssociated with increased inflammatory and anti-inflammatory responses. Studies by Sacheck and Blumberg (2001) showed that following prolonged exercise the number of circulating neutrophils increased, coupled with some features of an acute-phase inflammatory response such as an increase in pro-inflammatory cytokines, interleukin-1β (IL-1β), tumour necrosis factor (TNF-α) and interleukin-6 (IL-6) concentrations in plasma. Another study involving untrained subjects undertaking a 45min bicycle exercise and reaching 70% VO_2 max had also shown a significant

increase in the plasma levels of cytokines IL-1β, TNF-α and IL-6 (Vassilakopoulos et al., 2003). The supplementation with a mixture of antioxidants (vitamin E, A, C, allupurinol and N-acetylcysteine) reduced the production of IL-1β and IL-6 lower than the baseline. The pro-inflammatory cytokines IL-1β, TNF-α and IL-6 facilitate the influx of lymphocytes, neutrophils and monocytes into tissue after injury. Exercise is thought to activate phagocytotic processes to generate an oxidative burst of ROS such as superoxide ($^{\cdot}O_2^-$) which may aid in proteolysis of the injured cells/tissue.

Oxidative stress may also be caused by ischaemia-repurfusion. During exercise blood is shunted away from several organs to cover an increase in blood supply in active skeletal muscle leading to transient general tissue ischaemia (O_2 depletion) or hypoxia (O_2 concentration lower than normal) in these organs. In addition, exercise intensity exceeding VO_2 max leads to muscle fibre hypoxia as the oxygen supply fails to match the energy requirements (Koyama et al., 1999). This results in a fall in ATP levels, and degradation of AMP resulting in an accumulation of hypoxanthine (figure 4.1). As a result of the depletion of ATP the cell can no longer maintain a proper ion gradient across the membrane and the tissues are in a state of disruption. This results in a cytosolic build up of Ca^{2+} ions that activate Ca^{2+}-stimulated proteases. These proteases catalyse the proteolysis of xanthine dehydrogenase (XDH) to xanthine oxidase (XOD) (Roy and McCord, 1983). After exercise, reperfusion (reoxygenation) of the hypoxic tissues results in ROS generation, primarily in the form of superoxide ($^{\cdot}O_2^-$). The major source of superoxide appears to result from the action of XOD and its oxidation of hypoxanthine to uric acid (McCord and Fridovich, 1968; Roy and McCord, 1983). XOD in contrast to XDH, transfers electrons to molecular oxygen

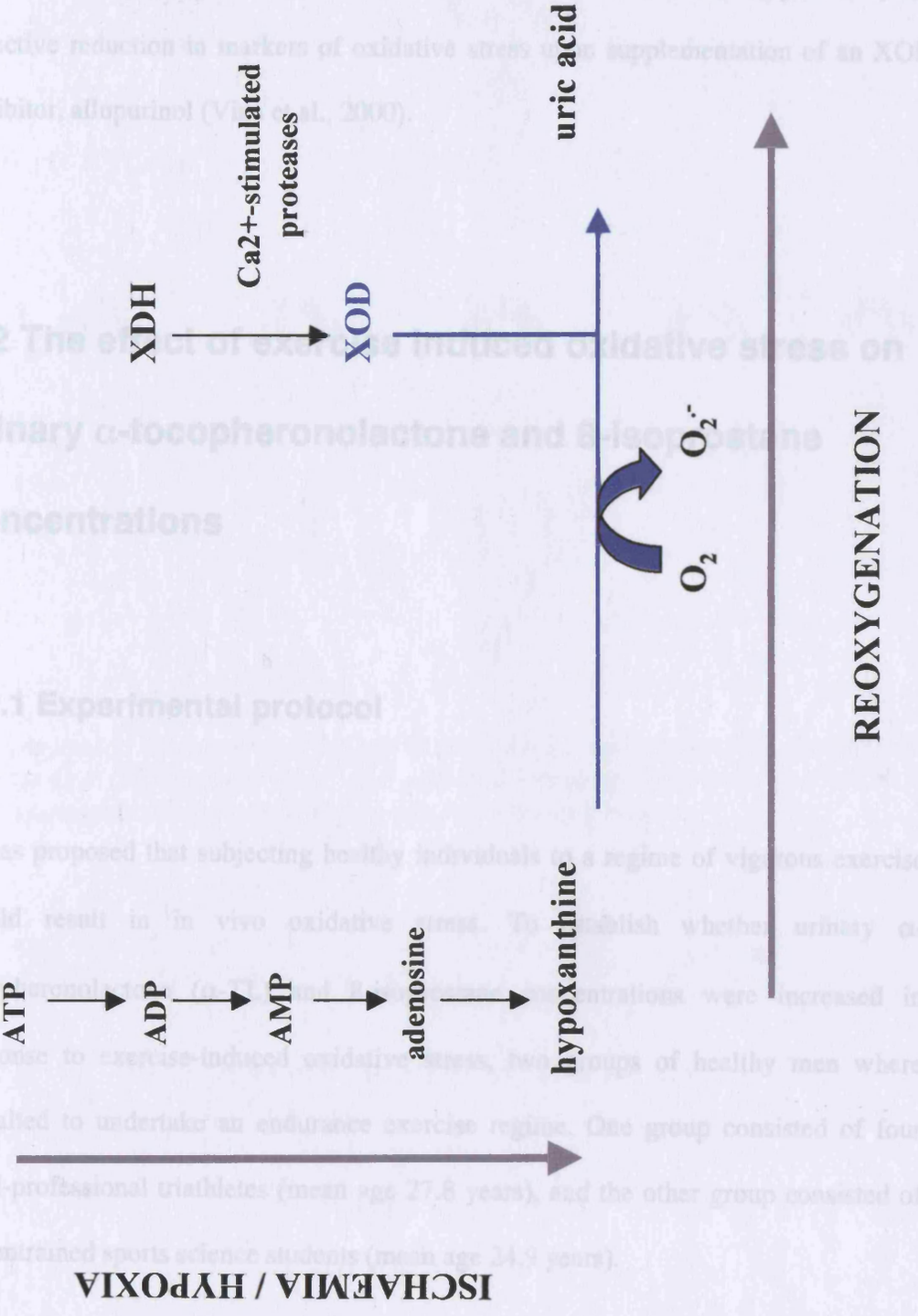


Figure 4.1; A proposed mechanism for the formation of ROS upon reoxygenation of ischaemic and hypoxic tissues

rapidly generating superoxide ($^{\cdot}O_2^-$) and hydrogen peroxide (H_2O_2) (McCord et al., 1985; Halliwell and Gutteridge, 1999).

The role of XOD in exercise-induced oxidative stress has been supported by an effective reduction in markers of oxidative stress upon supplementation of an XOD inhibitor, allupurinol (Vina et al., 2000).

4.2 The effect of exercise induced oxidative stress on urinary α-tocopheronolactone and 8-isoprostane concentrations

4.2.1 Experimental protocol

It was proposed that subjecting healthy individuals to a regime of vigorous exercise would result in in vivo oxidative stress. To establish whether urinary α-tocopheronolactone (α-TL) and 8-isoprostane concentrations were increased in response to exercise-induced oxidative stress, two groups of healthy men where recruited to undertake an endurance exercise regime. One group consisted of four semi-professional triathletes (mean age 27.8 years), and the other group consisted of ten untrained sports science students (mean age 24.9 years).

Both the triathlete and untrained groups took part in a five-day regime (figure 4.2), in which samples of the first morning void urine were collected each day. The subjects

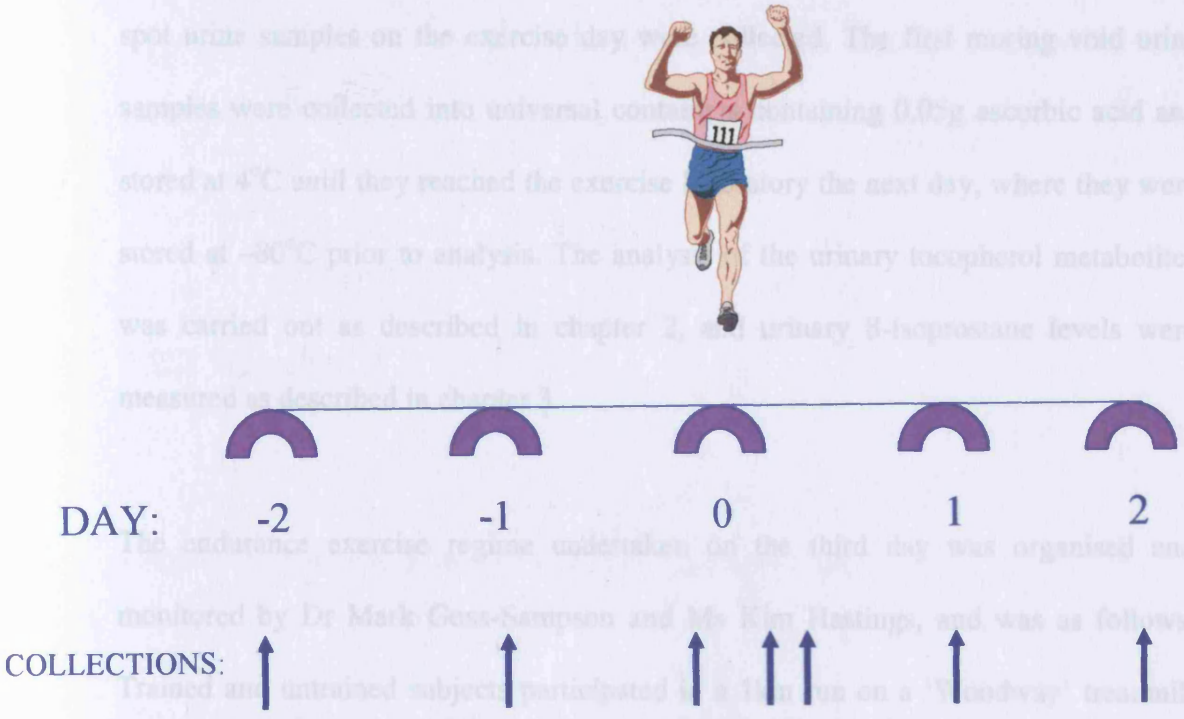


Figure 4.2; Study protocol for triathletes and untrained healthy men. Samples of the first morning void urine were collected including the exercise day and any subsequent urine spot samples on that day following exercise

did not train for the first two days (day -2 and -1) of the study. At about mid-day on the third day (day 0), they underwent an endurance exercise regime. Any subsequent spot urine samples on the exercise day were collected. The first moring void urine samples were collected into universal containers containing 0.05g ascorbic acid and stored at 4°C until they reached the exercise laboratory the next day, where they were stored at –80°C prior to analysis. The analysis of the urinary tocopherol metabolites was carried out as described in chapter 2, and urinary 8-isoprostane levels were measured as described in chapter 3.

The endurance exercise regime undertaken on the third day was organised and monitored by Dr Mark Goss-Sampson and Ms Kim Hastings, and was as follows. Trained and untrained subjects participated in a 1km run on a 'Woodway' treadmill (11mph max) at a pace that was reported as slightly uncomfortable. This was followed by a steady state sub-maximal test on a 'King' Cycle ergometer. Power output was increased incrementally for 4 minutes before maintaining a steady level for another 5 minutes. After this point, the resistance was ramped up sharply for one further minute before maintaining a steady level for the last 5 minutes. Heart rate was recorded after nine minutes and at the termination of the exercise. Gases were analysed using VacuMed Mini Vista and VO_2 values were calculated. Following the sub-maximal test, a ramped max-test was administered. This involved the subjects cycling at increasing intensities until volitional exhaustion using rating of perceived exertion (RPE) scores according to the Borg scale (Borg, 1998), which determines physical activity intensity by the total 'feeling' of the exercise by the subject. The Borg scale starts at 6 for no perceived exertion by the exerciser and increases to 20 for maximum exertion.

The measurements carried out in the laboratory to monitor activity intensities, showed that all the triathletes and untrained participants reached 90-100% maximum age-related heart rate and VO_2max, with RPE scores in the range of 15-20 on the Borg scale upon reaching exhaustion and termination of the exercise.

4.2.2 Results

4.2.2.1 Urinary 8-isoprostane and α-TL concentrations

The concentrations of urinary α-tocopheronolactone (α-TL) were measured as described in chapter 2, and the "actual" concentrations of α-TL were calculated by deducting the estimated artefactual concentration. The concentrations of α-TL (nmol/mmol creatinine) in the urine of the participants over the five day regime, were compared to the concentrations of urinary 8-isoprostane (pmol/mmmol creatinine) and concentrations of urinary creatinine (mmol/l).

Firstly the results from the group of triathletes will be discussed. Figure 4.3 shows the levels of α-TL and 8-isoprostane in the urine of each subject over the five day regime. α-TL concentrations for subjects 1-4 were in the range of 13-117 nmol/mmol creatinine, and 8-isoprostane concentrations were in the range 160-625 pmol/mmol creatinine. Only subject 3 showed an increase in both α-TL and 8-isoprostane

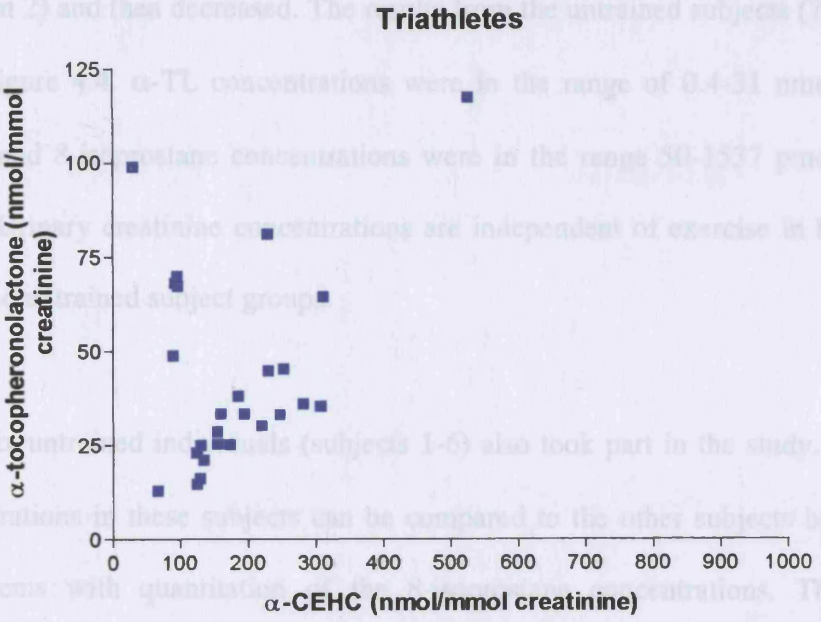


Figure 4.9a); The correlation of α-CEHC with α-TL for urine samples analysed (n=26) from the four triathlete subjects. [Spearman's r value =0.1904]

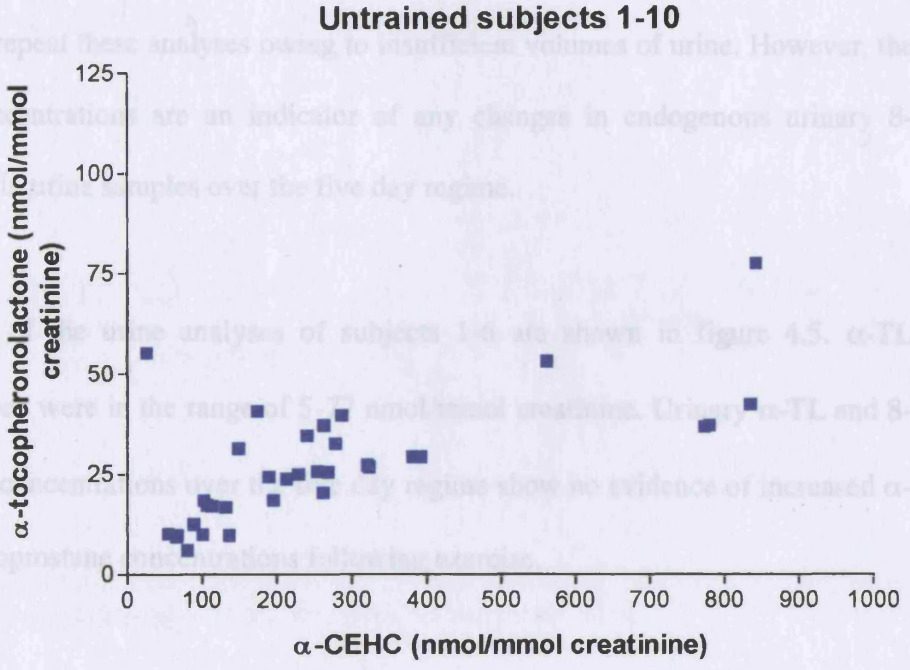


Figure 4.9b); The correlation of α-CEHC with α-TL for urine samples analysed (n=69) from untrained subjects 1-10. [Spearman's r=0.7187]

concentrations following exercise, which peaked on the same day as the exercise regime (0pm 2) and then decreased. The results from the untrained subjects (7-10) are shown in figure 4.4. α-TL concentrations were in the range of 0.4-31 nmol/mmol creatinine, and 8-isoprostane concentrations were in the range 50-1537 pmol/mmol creatinine. Urinary creatinine concentrations are independent of exercise in both the triathlete and untrained subject groups.

A further six untrained individuals (subjects 1-6) also took part in the study. The α-TL concentrations in these subjects can be compared to the other subjects but there were problems with quantitation of the 8-isoprostane concentrations. This was because the deuterated internal standard (d4-8-isoPGF$_{2\alpha}$) used for the quantitation of endogenous urinary 8-isoprostane, was incorrectly designated by the manufacturer and the correct concentration of the internal standard was unknown. It was not possible to repeat these analyses owing to insufficient volumes of urine. However, the relative concentrations are an indicator of any changes in endogenous urinary 8-isoprostane in urine samples over the five day regime.

The results of the urine analyses of subjects 1-6 are shown in figure 4.5. α-TL concentrations were in the range of 5-77 nmol/mmol creatinine. Urinary α-TL and 8-isoprostane concentrations over the five day regime show no evidence of increased α-TL and 8-isoprostane concentrations following exercise.

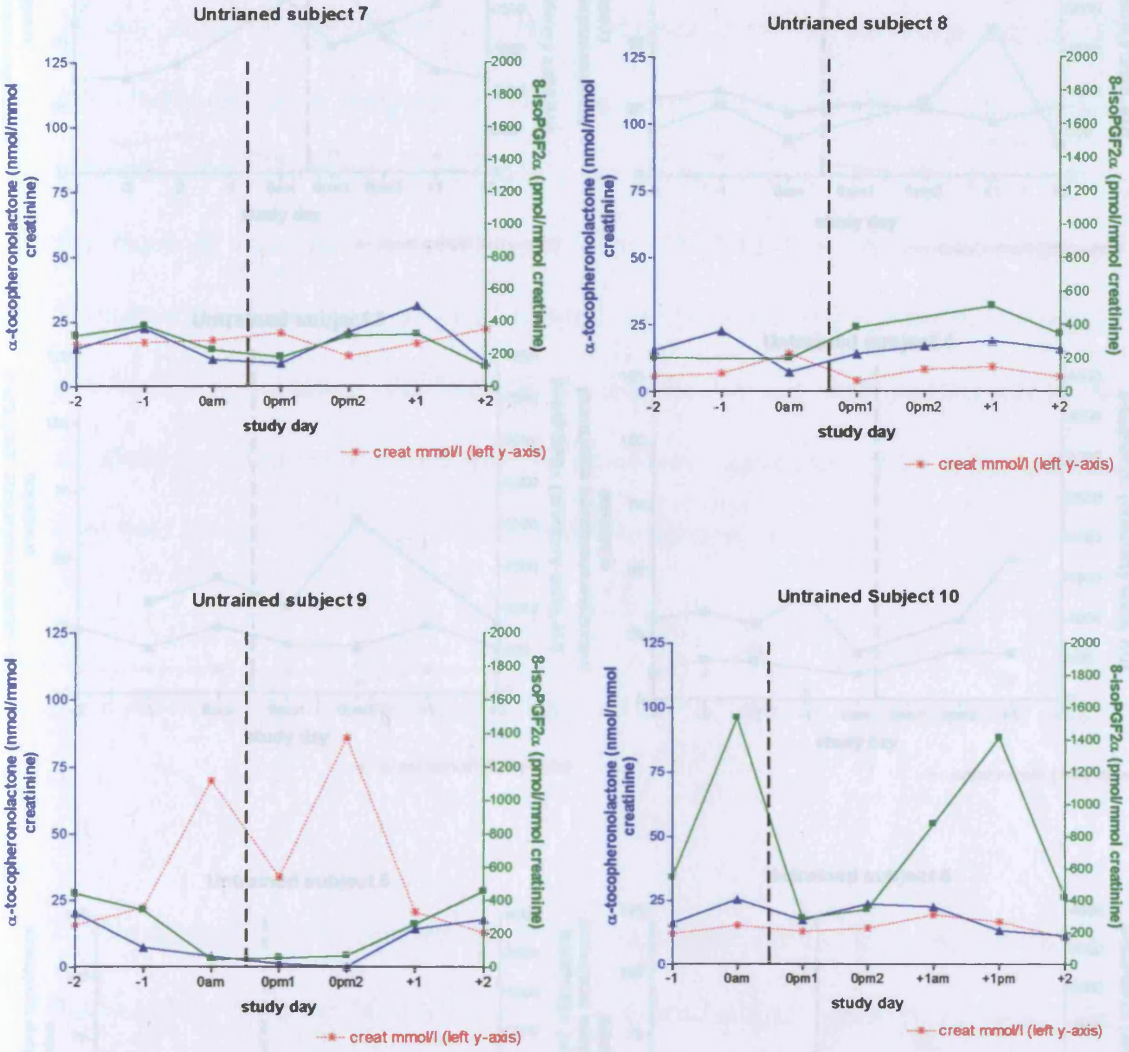


Figure 4.4; Urinary α-tocopheronolactone, 8-isoprostane and creatinine concentrations over the five-day study regime for the untrained subjects (7-10).

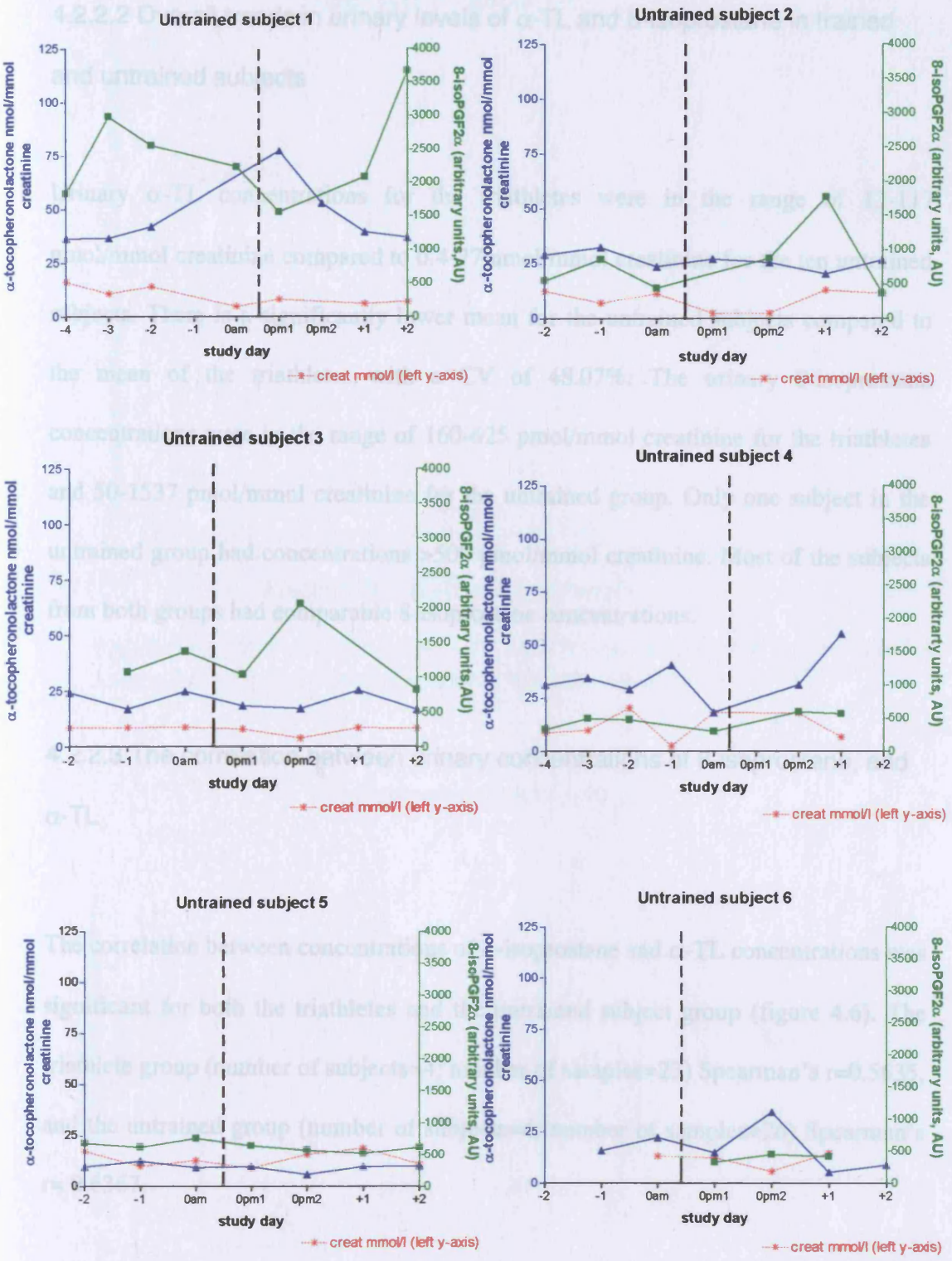


Figure 4.5; Urinary α-tocopheronolactone concentrations, 8-isoprostane levels and creatinine concentrations over the five day study regime for untrained subjects 1-6.

4.2.2.2 Overall trends in urinary levels of α-TL and 8-isoprostane in trained and untrained subjects

Urinary α-TL concentrations for the triathletes were in the range of 13-117 nmol/mmol creatinine compared to 0.4-77 nmol/mmol creatinine for the ten untrained subjects. There is a significantly lower mean for the untrained subjects compared to the mean of the triathletes with a CV of 48.07%. The urinary 8-isoprostane concentrations were in the range of 160-625 pmol/mmol creatinine for the triathletes and 50-1537 pmol/mmol creatinine for the untrained group. Only one subject in the untrained group had concentrations >500 pmol/mmol creatinine. Most of the subjects from both groups had comparable 8-isoprostane concentrations.

4.2.2.3 The correlation between urinary concentrations of 8-isoprostane, and α-TL

The correlation between concentrations of 8-isoprostane and α-TL concentrations was significant for both the triathletes and the untrained subject group (figure 4.6). The triathlete group (number of subjects=4; number of samples=23) Spearman's r=0.5635, and the untrained group (number of subjects=4; number of samples=26) Spearman's r= 0.6367.

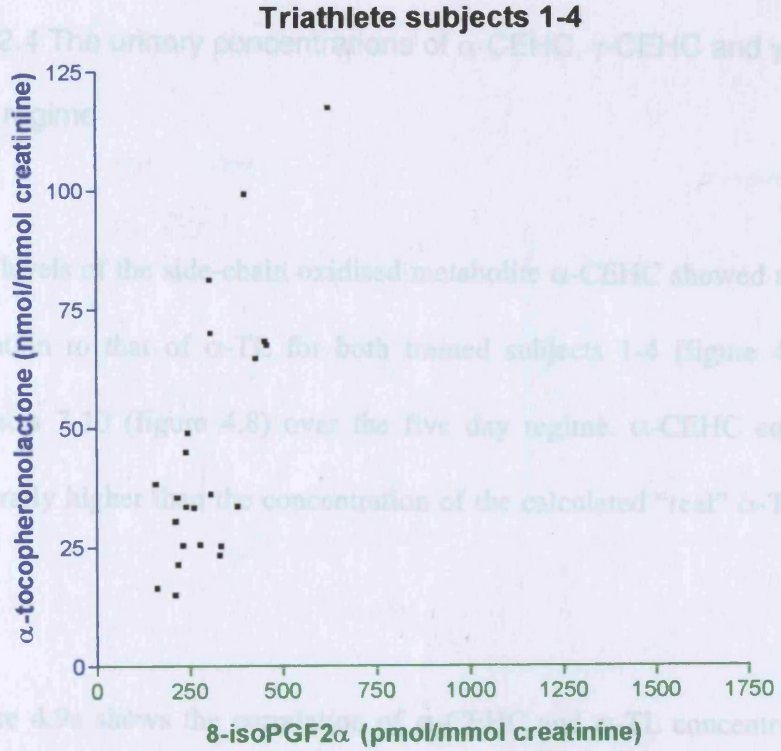


Figure 4.6a); The correlation of α-TL with 8-isoprostane for all urine samples analysed (n=26) from the four triathlete subjects. [Spearman's r=0.5635]

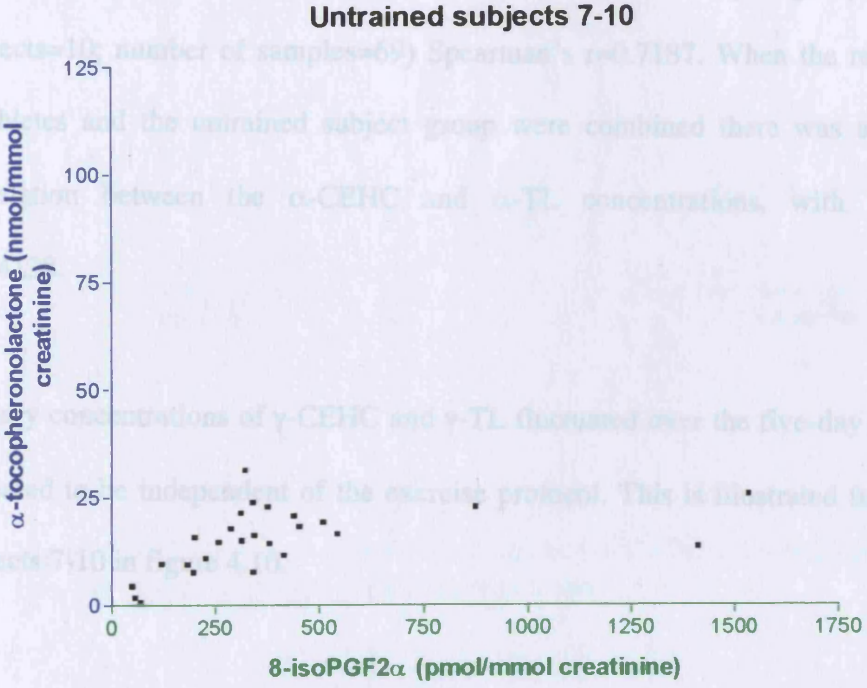


Figure 4.6b); The correlation of α-TL with 8-isoprostane for all urine samples analysed (n=28) from untrained subjects (7-10). [Spearman's r=0.6367]

4.2.2.4 The urinary concentrations of α-CEHC, γ-CEHC and γ-TL over the five day regime

The levels of the side-chain oxidised metabolite α-CEHC showed a similar pattern of variation to that of α-TL for both trained subjects 1-4 (figure 4.7) and untrained subjects 7-10 (figure 4.8) over the five day regime. α-CEHC concentrations were generally higher than the concentration of the calculated "real" α-TL by at least two-fold.

Figure 4.9a shows the correlation of α-CEHC and α-TL concentrations, which was not significant for the triathletes (number of subjects=4; number of samples=26, Spearman's r=0.1904). Figure 4.9b shows that the correlation of α-CEHC and α-TL concentrations was, however, significant for the untrained subject group (number of subjects=10; number of samples=69) Spearman's r=0.7187. When the results of the triathletes and the untrained subject group were combined there was a significant correlation between the α-CEHC and α-TL concentrations, with Spearman's r=0.4138.

Urinary concentrations of γ-CEHC and γ-TL fluctuated over the five-day regime and appeared to be independent of the exercise protocol. This is illustrated for untrained subjects 7-10 in figure 4.10.

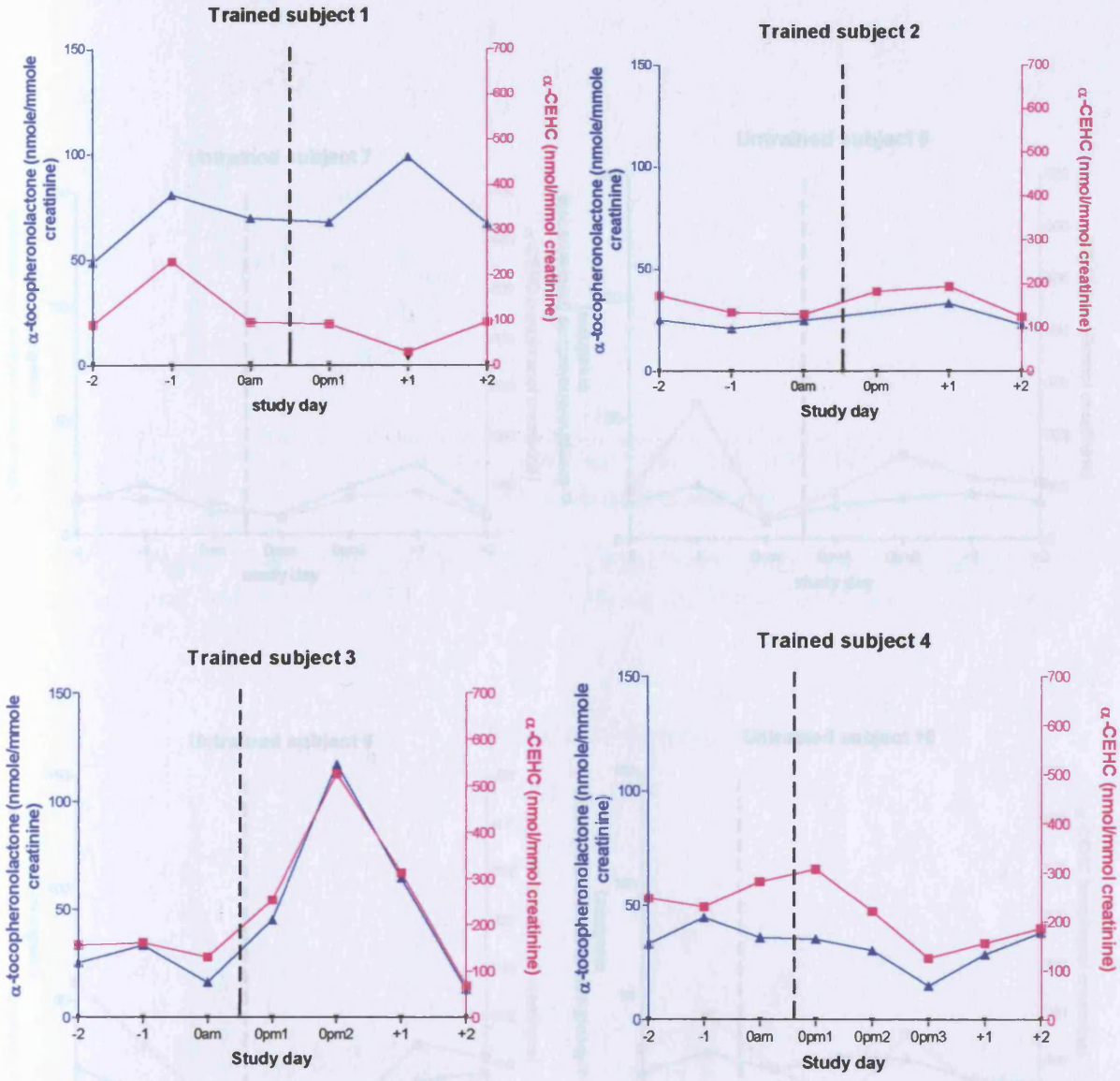


Figure 4.7; Urinary α-tocopheronolactone and α-CEHC concentrations over the five-day study regime for the triathlete subjects.

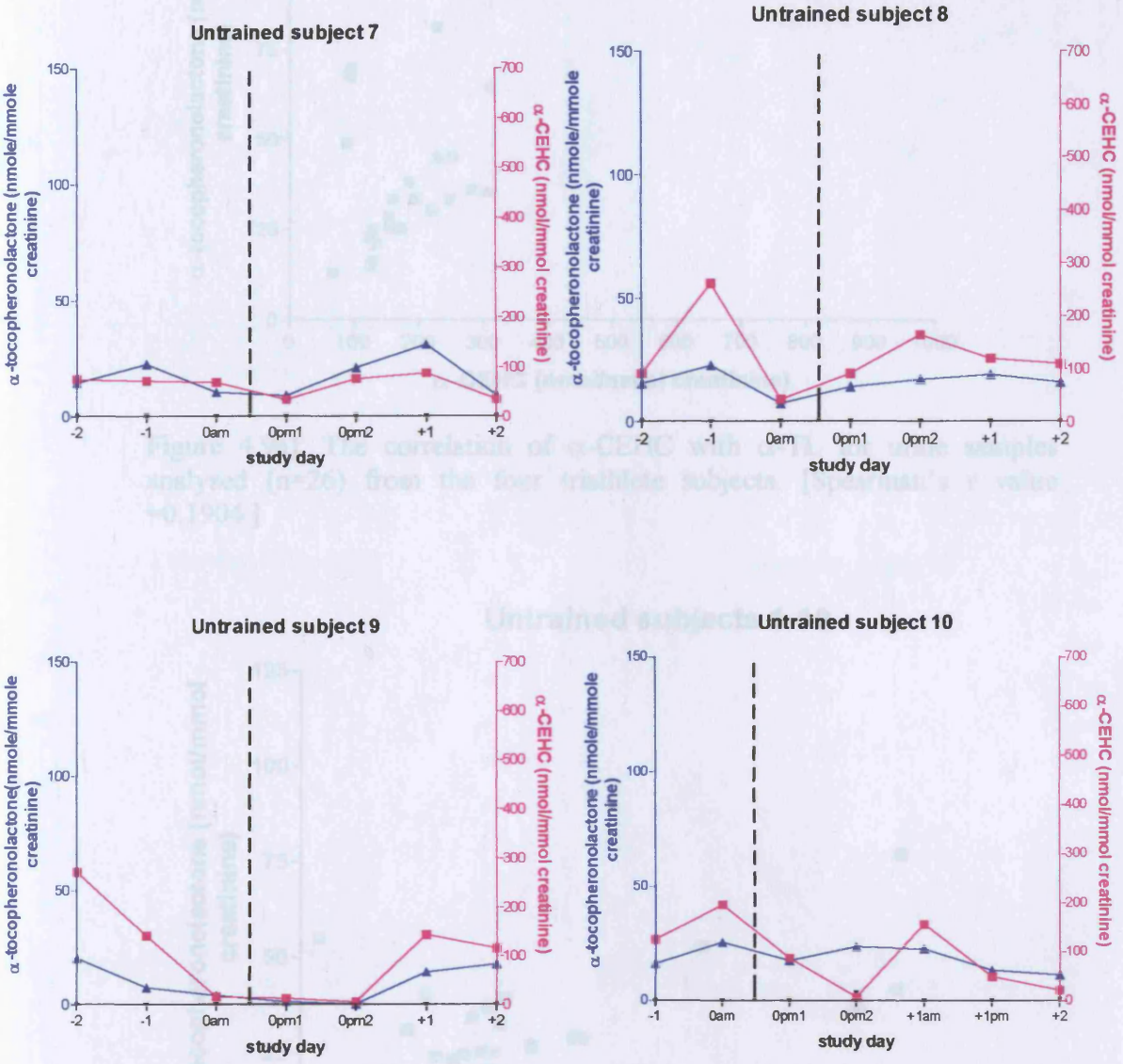


Figure 4.8; Urinary α-tocopheronolactone and α-CEHC concentrations over the five day study regime for untrained subjects 7-10

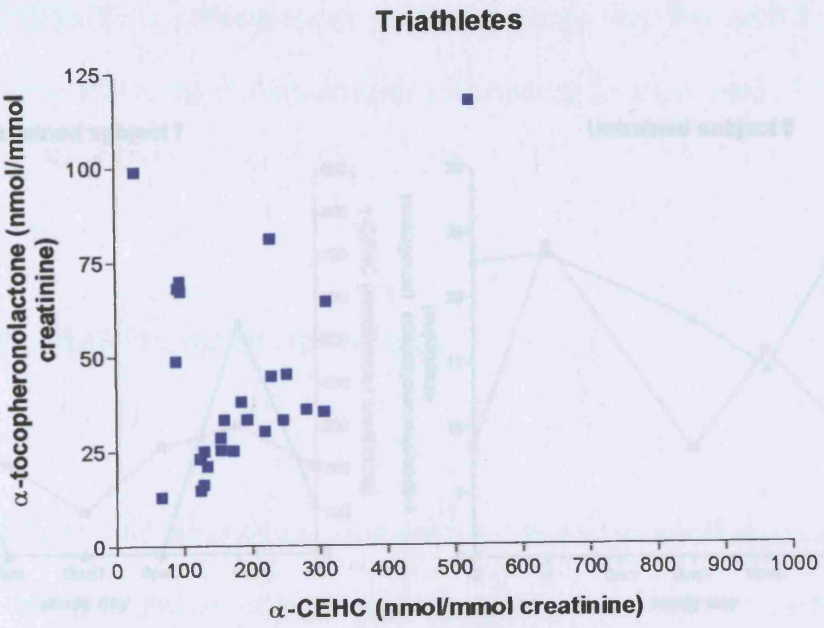


Figure 4.9a); The correlation of α-CEHC with α-TL for urine samples analysed (n=26) from the four triathlete subjects. [Spearman's r value =0.1904]

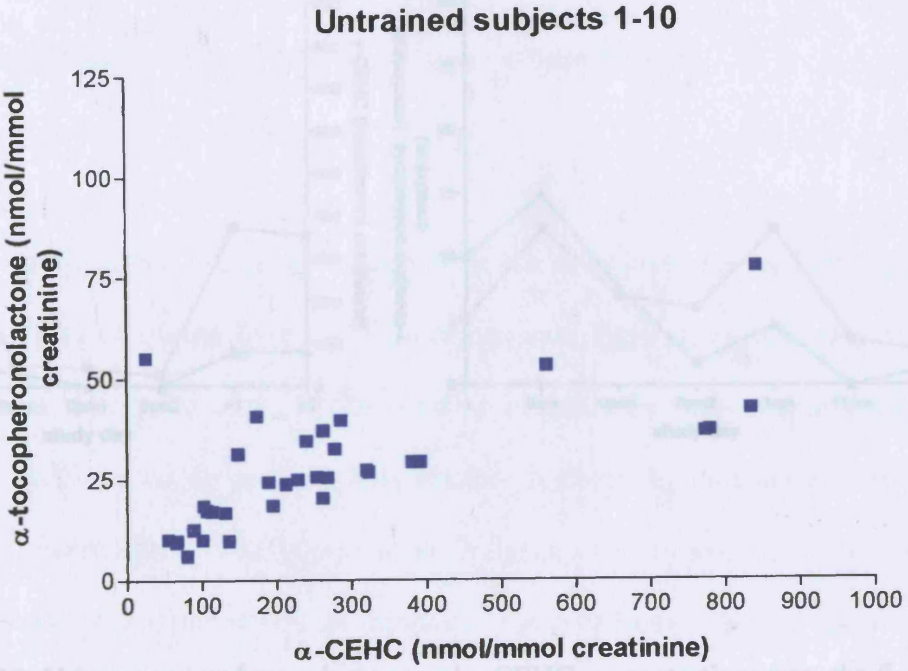


Figure 4.9b); The correlation of α-CEHC with α-TL for urine samples analysed (n=69) from untrained subjects 1-10. [Spearman's r=0.7187]

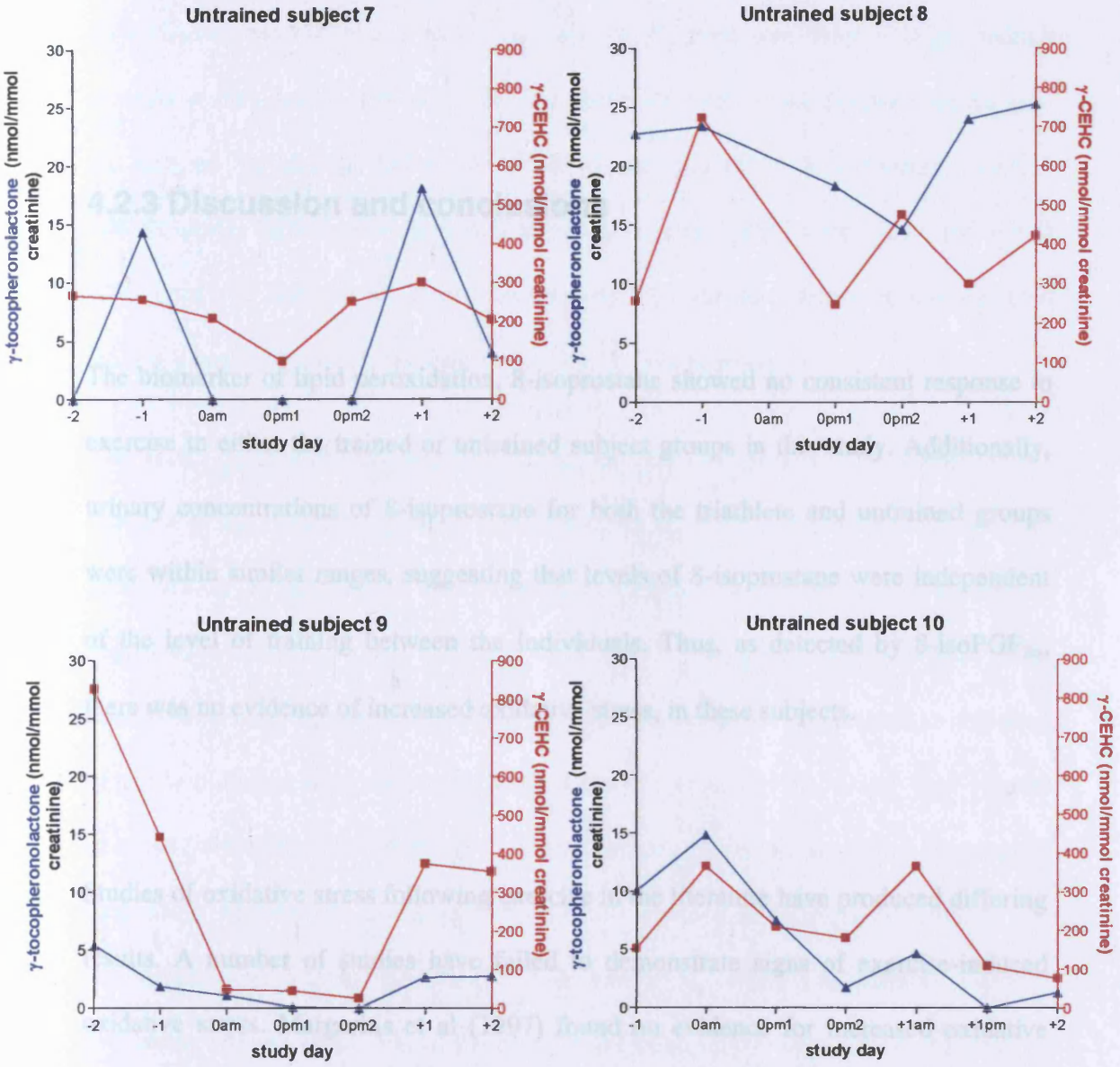


Figure 4.10; Urinary γ-tocopheronolactone and γ-CEHC concentrations over the five day study regime for untrained subjects 7-10.

It was not possible to accurately quantitate the concentrations of α-CMBHC and oxidised α-CMBHC as concentrations were consistently very low with a retention time towards the end of the chromatographic run resulting in broad peaks.

4.2.3 Discussion and conclusions

The biomarker of lipid peroxidation, 8-isoprostane showed no consistent response to exercise in either the trained or untrained subject groups in this study. Additionally, urinary concentrations of 8-isoprostane for both the triathlete and untrained groups were within similar ranges, suggesting that levels of 8-isoprostane were independent of the level of training between the individuals. Thus, as detected by 8-isoPGF$_{2\alpha}$, there was no evidence of increased oxidative stress, in these subjects.

Studies of oxidative stress following exercise in the literature have produced differing results. A number of studies have failed to demonstrate signs of exercise-induced oxidative stress. Margaritis et al (1997) found no evidence for increased oxidative stress in 12 triathletes taking part in a long distance triathlon, by measuring plasma glutathione concentrations (GSH), despite an inflammatory response detected by increased plasma leukocyte counts and neopterin concentrations. This led them to conclude that the level of training of this group of individuals made them less susceptible to oxidative stress. Witt et al (1992) showed that the urinary output of the oxidatively damaged RNA base 8-hydroxydeoxyguanosine (8-OHdG) was unaffected

by several sub-maximal exercise bouts. Lovlin et al (1987) found that where individuals underwent three different levels of exercise, which included VO_2max (high intensity running to exhaustion), 70%VO_2max (moderate intensity running) and 40%VO_2max (low intensity running), only at VO_2max was there a large enough increase in free radical production to overwhelm the antioxidant defences resulting in an increase in plasma MDA. At 70%VO_2max no affect was detected and at 40%VO_2max, MDA levels decreased to below baseline. Studies by Mastaloudis et al (2001) showed that eleven athletes undergoing a 50 km ultramarathon, had elevated plasma 8-isoPGF$_{2\alpha}$ which returned to baseline 24 hours post race.

The effect of exercise on oxidative stress appears to depend on the intensity and duration of the exercise regime (Shern-Brewer et al., 1998; Chevion et al., 2003; Liu et al., 2000), and appears to affect muscle and heart to a different extent than other organs such as the brain and liver (Liu et al., 2000). The general effect of short-term intense exercise (acute) is an elevation of oxidative stress biomarkers and an overload of the antioxidant defence system (Shern-Brewer et al., 1998). Long term regular exercise (chronic) tends, however, to induce an adaptive response, with a decrease in biomarkers of oxidative stress as a result of an increase in antioxidant defences and the upregulation of repair systems (Shern-Brewer et al., 1998). The adaptive response in humans (Asami et al., 1998) and rats (Radak et al., 2002) depends on the level of training of the individual taking part in an exercise regime. It has been proposed that training-induced adaptation was able to induce accelerated oxygen uptake and enhanced ATP/AMP stability which is critical for cellular energy supply and demand and thus cell homeostasis (Korzeniewski and Zoladz, 2003). Exercise has been shown to induce an increase in antioxidant enzyme activity, including superoxide dismutase and catalase activities (Wozniak et al., 2001; Tauler et al., 1999; Jenkins, 1988).

Svensson et al (2002) reported that the redox balance of reduced glutathione (GSH), and its oxidised form (GSSG) in skeletal muscle showed a rapid adaptive response to repeated bouts of strenuous exercise with a reduced level of GSSG.

Because only one measure of oxidative stress, urinary concentrations of 8-isoprostane, was used and there were no consistent changes following exercise it was not possible to determine whether α-TL could be used as a biomarker of in vivo oxidative stress. The fact that there was a significant correlation between urinary α-TL and 8-isoprostane concentrations suggests that α-TL could potentially be an effective lipid peroxidation biomarker, but further data is necessary.

When combining all the data α-CEHC and α-TL concentrations showed a significant correlation and it is possible that α-TL concentrations merely reflect the concentrations of α-CEHC.

In summary, despite the present study being designed to take into account parameters such as exercise intensity, level of training of the individuals and the fact that some oxidation products are elevated hours or even days following exercise, no consistent response was detected in the urinary lipid peroxidation marker, 8-isoprostane. Although urine samples were collected up to two days following the day of exercise, it may be that this marker was only elevated after this period, as Sacheck et al (2003) reported that plasma total F_2-isoprostane (four different isomers including $PGF_{2\beta}$, 8-iso$PGF_{2\alpha}$, $PGF_{2\alpha}$ and trans-$PGF_{2\alpha}$) concentrations only showed an increase 72 hours post exercise.

In retrospect, exercise is a complex situation involving a wide range of physiological and biochemical responses, including biochemical adaptation (Radak et al., 2002), and may have been an inappropriate group to study. Further work carried out by the vitamin E research group at the Institute of Child Health, London, has suggested that children with sepsis might have been a better group to study.

Chapter 5

In vivo investigation of the cellular localisation of tocopherol metabolism

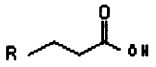
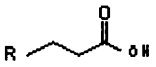
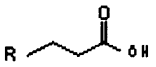
5.1 Cellular localisation and mechanism of β-oxidation of fatty acids

The saturated phytyl side-chain of the tocopherols is identical to that of the fatty acid phytanic acid (see section 5.1.4), it is plausible that tocopherols may follow the same metabolic pathway. Fatty acids are normally metabolised in the mitochondria and peroxisomes and this will now be briefly discussed.

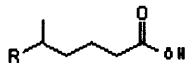
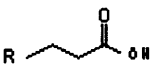
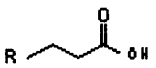
5.1.1 The metabolism of fatty acids

Fatty acids (FAs) are mainly oxidised by β-oxidation, a series of reactions that are carried out in mitochondria and peroxisomes. The two intracellular organelles play distinct roles in the metabolism of FAs in the cell, as the substrate specificities differ between the two organelles (figure 5.1). The substrates for mitochondrial β-oxidation are the acyl-CoAs of short, medium and long-straight chain FAs. These FAs comprise the bulk of FAs derived from the diet. The substrates for peroxisomal β-oxidation are the acyl-CoAs of the less common FAs comprising a) the branched chain FAs, long-chain fatty acids (LCFA), very-long-chain fatty acids (VLCFAs) and 2-methyl-branched FAs like pristanic acid, b) bile acid intermediates, dihydroxycholestanoic acid (DHCA) and trihydroxycholestanoic acid (THCA) and c) some eicosanoids such as the leukotrienes and prostaglandins.

Mitochondrial substrates

short chain fatty acids (SCFAs)	medium chain fatty acids (MCFAs)	long chain fatty acids (LCFAs)
		
P= 2-4C	R= 4-12C	R= 12-20C

Peroxisomal substrates

branched chain fatty acids	long chain fatty acids (LCFAs)	very long chain fatty acids (VLCFAs)
		
	R= 12-20C	R>20C

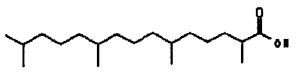
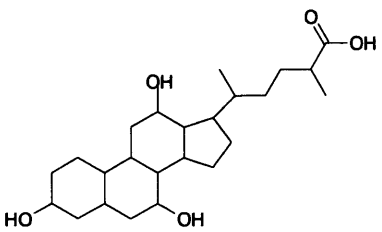
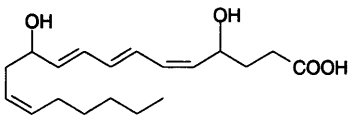
2-methyl branched fatty acids e.g. pristanic acid	bile acid intermediates e.g.trihydroxycholestanoic acid (THCA)	some eicosanoids e.g. leukotrienes (LTB4)
		

Figure 5.1; Substrates of mitochondrial and peroxisomal oxidation

Mitochondrial and peroxisomal β-oxidation of FAs involves four basic reactions: dehydrogenation, hydration, dehydrogenation, and finally thiolytic cleavage. In general the enzymes involved in these steps differ between the two organelles and are encoded by distinct genes. There are, however, a few exceptions where the enzymes in both compartments are products of the same genes. The mitochondria are responsible for the complete β-oxidation of FAs to acetyl-CoA units, which then enter the tricarboxylic acid (TCA) cycle. This results in the production of NADH and $FADH_2$ which are energy rich molecules that can be used to produce ATP during the process of oxidative phosphorylation.

The peroxisomes tend to be involved in chain-shortening of substrates, after which the products are exported to the mitochondria for further β-oxidation. Although the peroxisomes also contain flavoproteins, electrons are donated to molecular oxygen (O_2), with the formation of hydrogen peroxide (H_2O_2). Hydrogen peroxide is converted to molecular oxygen and water by a peroxisomal catalase. The peroxisomal pathway is only half as efficient in the production of energy in the form of ATP compared to the mitochondrial oxidation of FAs.

5.1.2 Role of mitochondria in fatty acid oxidation

FAs have to be converted to coenzyme A (CoA) esters at the outer mitochondrial membrane and transported across the inner mitochondrial membrane as carnitine esters prior to β-oxidation. The three enzymes involved in the carnitine dependent

transport system include; carnitine-palmitoyl transferase I (CPT-I), carnitine:acyl carnitine translocase (CACT) and carnitine–palmitoyl transferase II (CPT-II). The fatty acid acyl-CoAs are converted to acyl-carnitines by CPT-I, located in the outer mitochondrial membrane (Hoppel et al., 1998). These acyl-carnitines are then translocated from the inner membrane space to the mitochondrial matrix by an exchange reaction catalyzed by CACT, which is an inner mitochondrial membrane protein. Upon entry into the mitochondrial matrix the acyl-carnitines are reconverted to their respective acyl-CoAs by CPT-II, located in the inner-leaflet of the mitochondrial inner-membrane. CPT-I was demonstrated to be the 'rate-limiting' step of fatty acid β-oxidation flux by inhibition studies of CPT-I with malonyl-CoA (McGarry and Foster, 1980).

Once acyl-CoA is regenerated in the mitochondrial matrix, it enters into the β-oxidation reaction pathway. The pathway of β-oxidation is shown in figure 5.2. The first step involves the formation of 2-enoyl-CoA from its corresponding saturated ester by acyl-CoA dehydrogenase which is dependent on electron transfer flavoprotein (ETF), a matrix FAD-linked protein. Four different acyl-CoA dehydrogenases have been reported with different but overlapping substrate chain-length specificities; short-chain acyl-CoA dehydrogenase (SCAD) (C4-C6), medium-chain acyl-CoA dehydrogenase (MCAD) (C4-C20), long-chain acyl-CoA dehydrogenase (LCAD) (C8-C20), and very long-chain acyl-CoA dehydrogenase (VLCAD) (C12-C24). The resulting 2-enoyl-CoAs are then hydrated to 3-hydroxyacyl-CoA by 2-enoyl-CoA hydratases. The three enzymes present in the mitochondria responsible for these catalytic reactions are short, medium and long-chain 2-enoyl-CoA hydratases. The dehydrogenation of 3-hydroxyacyl-CoA, the third

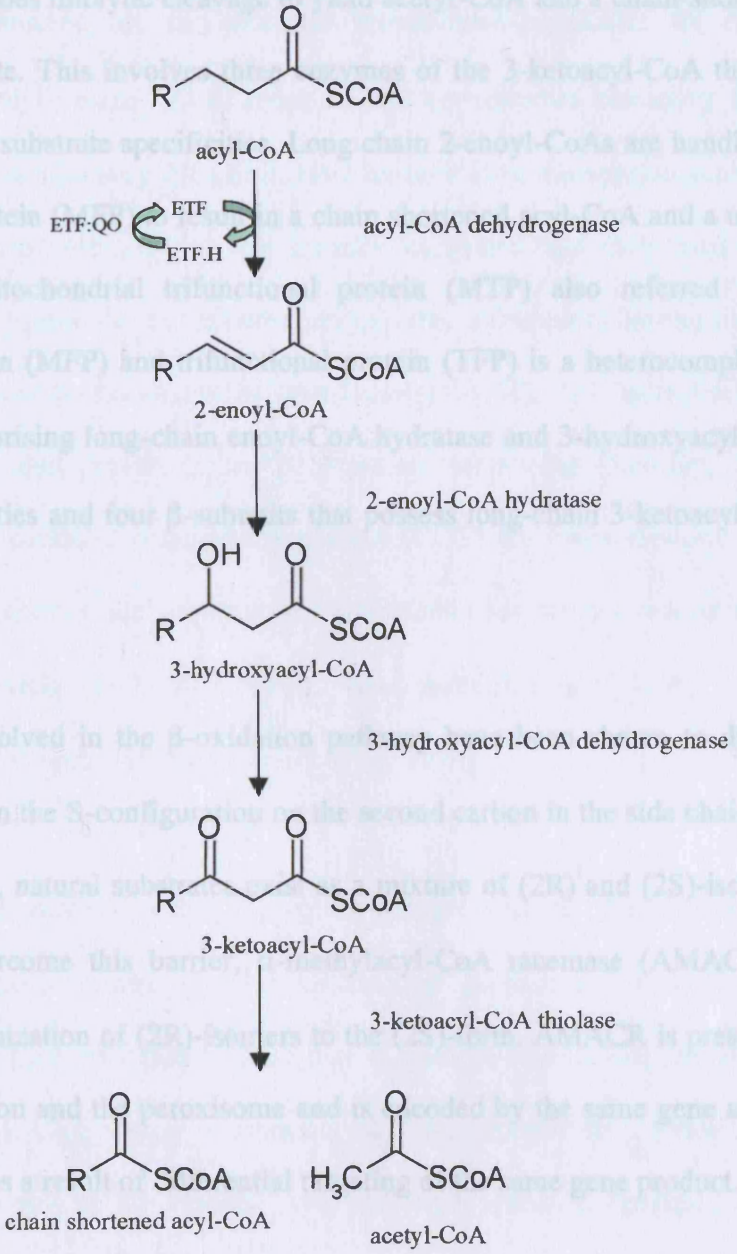


Figure 5.2; Mitochondrial β-oxidation of fatty acyl-CoAs
[ETF= electron transfer flavoprotein]

step, is carried out by two enzymes (3-hydroxyacyl-CoA dehydrogenases) with over-lapping chain length specificities to form the 3-ketoacyl-CoA intermediate. Finally, 3-ketoacyl-CoA undergoes thiolytic cleavage to yield acetyl-CoA and a chain-shortened acyl-CoA intermediate. This involves three enzymes of the 3-ketoacyl-CoA thiolase family with different substrate specificities. Long chain 2-enoyl-CoAs are handled by a multifunctional protein (MFP) to result in a chain shortened acyl-CoA and a unit of acetyl-CoA. The mitochondrial trifunctional protein (MTP) also referred to as mutifunctional protein (MFP) and trifunctional protein (TFP) is a heterocomplex of four α-subunits comprising long-chain enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities and four β-subunits that possess long-chain 3-ketoacyl-CoA thiolase activity.

All the enzymes involved in the β-oxidation pathway have been shown to display substrate specificity in the S-configuration on the second carbon in the side chain (see figure 5.4). However, natural substrates exist as a mixture of (2R) and (2S)-isomers, and in order to overcome this barrier, α-methylacyl-CoA racemase (AMACR) is involved in the racemization of (2R)-isomers to the (2S)-form. AMACR is present in both the mitochondrion and the peroxisome and is encoded by the same gene and its bimodal distribution is a result of differential targeting of the same gene product.

5.1.3 Role of peroxisomes in fatty acid oxidation

Although the mitochondria are the principal intracellular organelles for FA β-oxidation, Cooper and Beevers (1969) reported that peroxisomes can carry out β-oxidation of FAs in a similar way. However, the FAs have to be transported across the peroxisomal membrane. Although it was initially suggested that fatty acyl-CoAs could diffuse freely across the peroxisomal membrane, subsequent immunological studies showed that carnitine:acylcarnitine translocase (CACT), first characterised in the mitochondria, is also present in the peroxisomal membrane (Ramsay, 2000). Additionally, human carnitine octanoyl transferase (COT) has been reported to be localised in the peroxisome and shown to be responsible for the conversion of the end-product of pristanic acid β-oxidation, 4,8-dimethylnonanoyl-CoA, for its transport into the mitochondria (Ferdinandusse et al., 1999).

The first step in peroxisomal β-oxidation involves dehydrogenation of acyl-CoA to 2-trans-enoyl-CoA (figure 5.3), which is the "rate-limiting" reaction and is catalyzed by FAD-dependent acyl-CoA oxidases (ACO) that reduces molecular oxygen (O_2) to hydrogen peroxide (H_2O_2), which is subsequently decomposed by catalase. Two forms of ACO are present in humans with different substrate specificities; 1) palmitoyl-CoA oxidase (also known as straight-chain acyl-CoA oxidase (SCOX)), which acts on both straight and branched-chain saturated fatty acyl-CoAs in rat peroxisomes but only on straight-chain acyl-CoAs in human peroxisomes and 2) branched-chain acyl-CoA oxidase (BCOX), which acts on

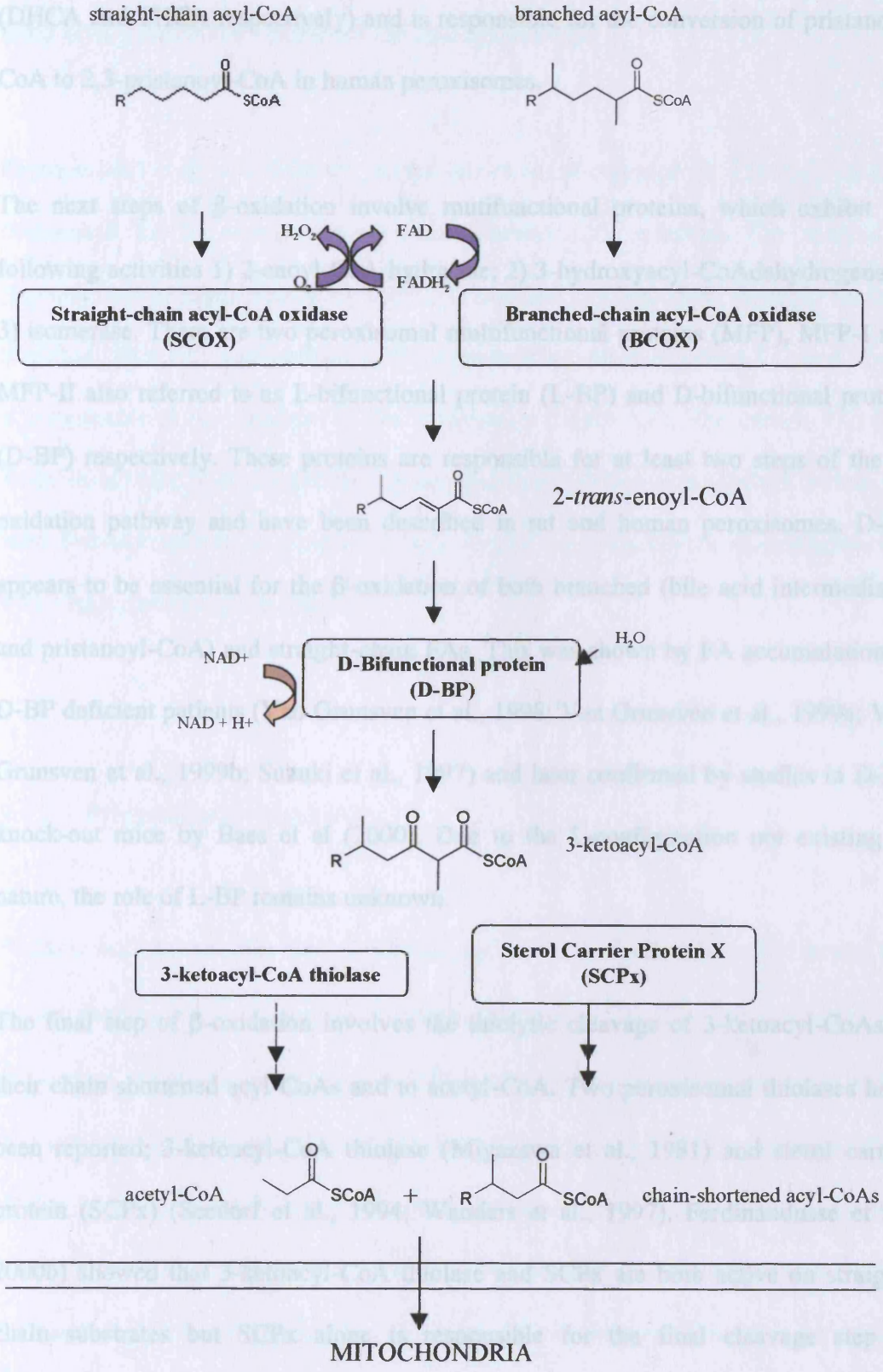


Figure 5.3; Steps involved in the oxidation of straight-chain very long chain fatty acids (VLCFAs) and branched chain fatty acids (e.g.pristanic acid).

substrates such as di-hydroxycoprostanoyl-CoA and tri-hydroxycoprostanoyl-CoA (DHCA and THCA respectively) and is responsible for the conversion of pristanoyl-CoA to 2,3-pristenoyl-CoA in human peroxisomes.

The next steps of β-oxidation involve mutifunctional proteins, which exhibit the following activities 1) 2-enoyl-CoA hydratase; 2) 3-hydroxyacyl-CoAdehydrogenase; 3) isomerase. There are two peroxisomal multifunctional proteins (MFP), MFP-I and MFP-II also referred to as L-bifunctional protein (L-BP) and D-bifunctional protein (D-BP) respectively. These proteins are responsible for at least two steps of the β-oxidation pathway and have been described in rat and human peroxisomes. D-BP appears to be essential for the β-oxidation of both branched (bile acid intermediates and pristanoyl-CoA) and straight-chain FAs. This was shown by FA accumulation in D-BP deficient patients (Van Grunsven et al., 1998; Van Grunsven et al., 1999a; Van Grunsven et al., 1999b; Suzuki et al., 1997) and later confirmed by studies in D-BP knock-out mice by Baes et al (2000). Due to the L-configuration not existing in nature, the role of L-BP remains unknown.

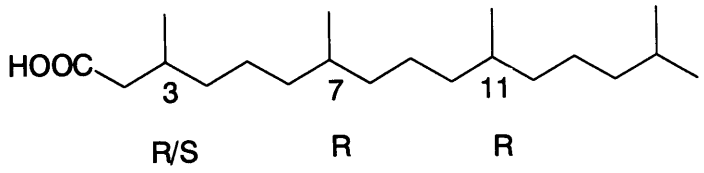
The final step of β-oxidation involves the thiolytic cleavage of 3-ketoacyl-CoAs to their chain shortened acyl-CoAs and to acetyl-CoA. Two peroxisomal thiolases have been reported; 3-ketoacyl-CoA thiolase (Miyazawa et al., 1981) and sterol carrier protein (SCPx) (Seedorf et al., 1994; Wanders et al., 1997). Ferdinandusse et al., 2000b) showed that 3-ketoacyl-CoA thiolase and SCPx are both active on straight-chain substrates but SCPx alone is responsible for the final cleavage step in β–oxidation of branched-chain substrates including pristanic acid and the bile acid intermediates DHCA and THCA (Ferdinandusse et al., 2001).

5.1.4 The cellular metabolism of phytanic and pristanic acids and the possible relation to tocopherols

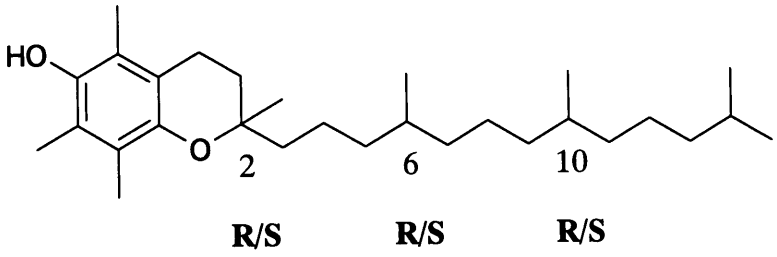
Phytanic acid is derived from the phytyl side-chain of chlorophyll. The absorption of chlorophyll by the human digestive tract, however, is minimal. The sources of phytanic acid in humans are from dairy products and ruminant fats. Phytanic acid is a branched chain fatty acid consisting of 3 asymmetric centres at carbon 3, 7 and 11 and is comparable to the structure of the tocopherols (figure 5.4). The carbon 7 and 11 atoms exist in the R-configuration (D-configuration). However, the carbon 3 atom has been demonstrated to be present in both R- and S-configurations (L-configuration) with varying relative proportions.

5.1.4.1 Phytanic acid metabolism

Phytanic acid cannot enter the β-oxidation spiral directly due to a β-methyl group, and it must therefore undergo prior α-oxidation, followed by decarboxylation to remove a one carbon moiety (Verhoeven et al., 1997a). Prior to α-oxidation, phytanic acid is first activated to phytanoyl-CoA by phytanoyl-CoA ligase (Pahan and Singh, 1995) (figure 5.5). Phytanoyl-CoA then enters the peroxisome by an undefined mechanism, which appears to involve active transport (Poulos et al., 1985; Singh et al., 1993; Jansen et al., 1997 and Jansen et al., 1996), where it undergoes α-oxidation (Avigan et al., 1966; Eldjarn et al., 1966). The first step of α-oxidation is its conversion to 2-hydroxyphytanoyl-CoA, by phytanoyl-CoA hydroxylase (PhyH). 2-

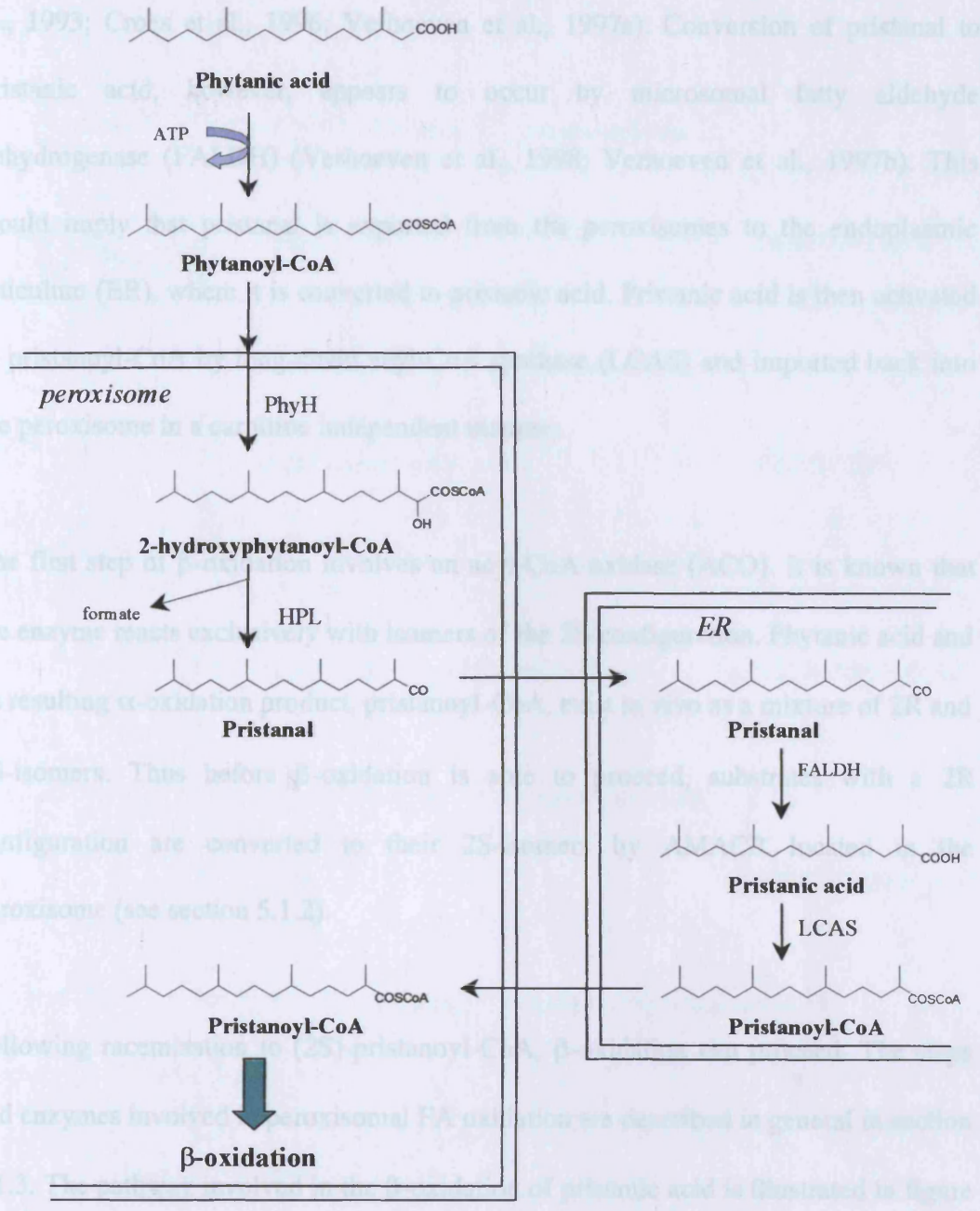


Phytanic acid



α-Tocopherol

Figure 5.4; The structure of Phytanic acid and its asymetric centres compared to α-tocopherol.



HPL: hydroxyphytanoyl-lyase
FALDH: fatty aldehyde dehydrogenase
LCAS: long chain acyl-CoA synthase

Figure 5.5; The α-oxidation pathway of phytanic acid, prior to β-oxidation

hydroxyphytanoyl-CoA then undergoes decarboxylation by 2-hydroxyphytanoyl-lyase (HPL), to form pristanal, releasing a one carbon compound, formate (Poulos et al., 1993; Croes et al., 1996; Verhoeven et al., 1997a). Conversion of pristanal to pristanic acid, however, appears to occur by microsomal fatty aldehyde dehydrogenase (FALDH) (Verhoeven et al., 1998; Verhoeven et al., 1997b). This would imply that pristanal is exported from the peroxisomes to the endoplasmic reticulum (ER), where it is converted to pristanic acid. Pristanic acid is then activated to pristanoyl-CoA by long-chain acyl-CoA synthase (LCAS) and imported back into the peroxisome in a carnitine independent manner.

The first step of β-oxidation involves an acyl-CoA oxidase (ACO). It is known that the enzyme reacts exclusively with isomers of the 2S-configuration. Phytanic acid and its resulting α-oxidation product, pristanoyl-CoA, exist in vivo as a mixture of 2R and 2S-isomers. Thus before β-oxidation is able to proceed, substrates with a 2R configuration are converted to their 2S-isomers by AMACR located in the peroxisome (see section 5.1.2).

Following racemisation to (2S)-pristanoyl-CoA, β-oxidation can proceed. The steps and enzymes involved in peroxisomal FA oxidation are described in general in section 5.1.3. The pathway involved in the β-oxidation of pristanic acid is illustrated in figure 5.6. Firstly, branched-chain acyl-CoA oxidase (BCOX) converts pristanoyl-CoA to 2,3-pristenoyl-CoA utilising flavin adeninenucleotide (FAD). The next two steps involve D-BP (MFP-II), responsible for the conversion of 2,3-pristenoyl-CoA to 3-ketopristanoyl-CoA via 3-hydroxypristanoyl-CoA. These two steps involve firstly hydration by 2-enoyl-CoA hydratase activity, followed by dehydrogenation by 3-

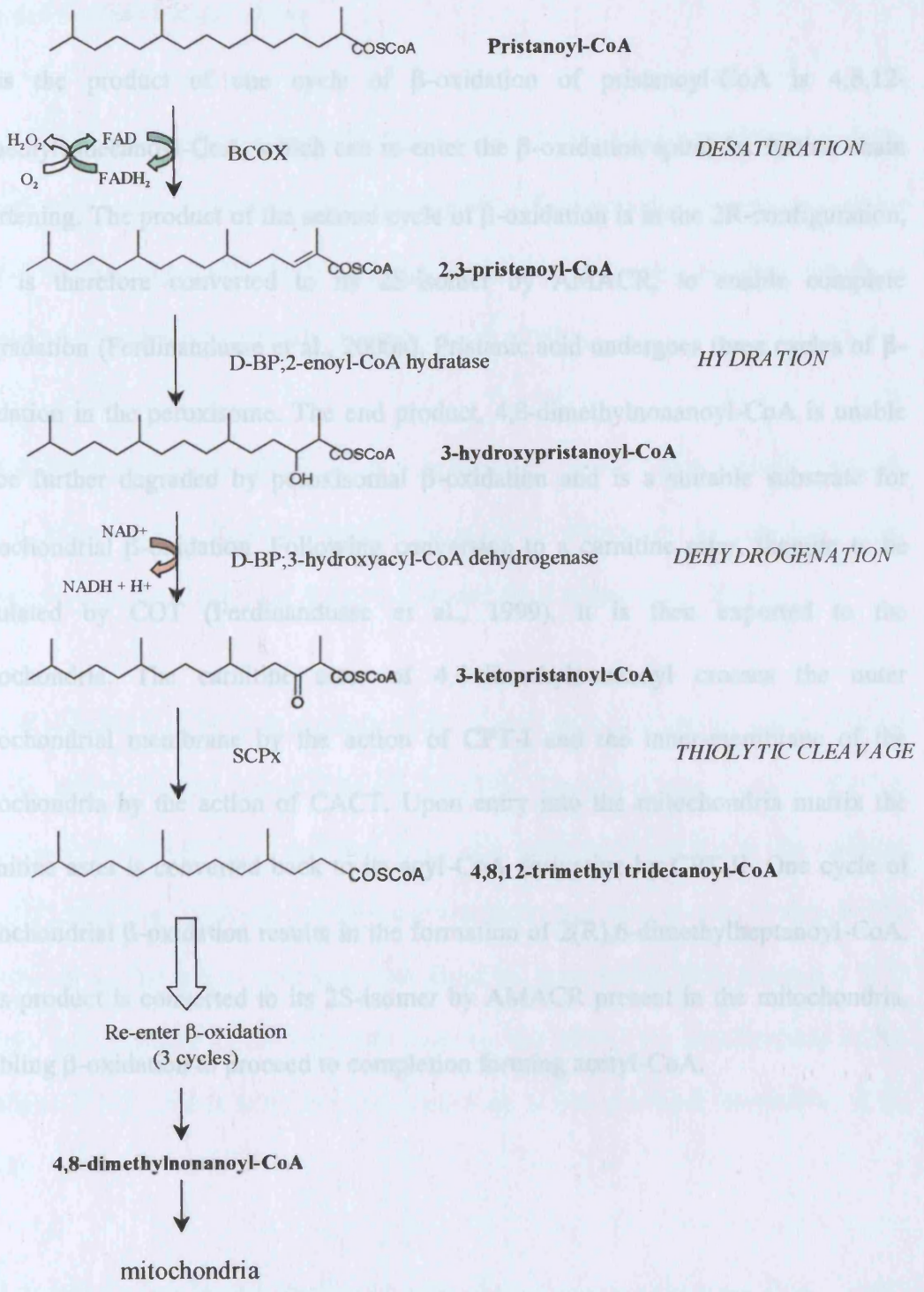


Figure 5.6; The peroxisomal β-oxidation of Pristanoyl-CoA

hydroxyacyl-CoA dehydrogenase activity. The final step of β-oxidation involves thiolytic cleavage of 3-ketopristanoyl-CoA by (SCPx) to form 4,8,12-trimethyltridecanoyl-CoA (Wanders et al., 1997).

Thus the product of one cycle of β-oxidation of pristanoyl-CoA is 4,8,12-trimethyltridecanoyl-CoA, which can re-enter the β-oxidation spiral for further chain shortening. The product of the second cycle of β-oxidation is in the 2R-configuration, and is therefore converted to its 2S-isomer by AMACR, to enable complete degradation (Ferdinandusse et al., 2000a). Pristanic acid undergoes three cycles of β-oxidation in the peroxisome. The end product, 4,8-dimethylnonanoyl-CoA is unable to be further degraded by peroxisomal β-oxidation and is a suitable substrate for mitochondrial β-oxidation. Following conversion to a carnitine ester, thought to be regulated by COT (Ferdinandusse et al., 1999), it is then exported to the mitochondria. The carnitine ester of 4,8-dimethylnonanoyl crosses the outer mitochondrial membrane by the action of CPT-I and the inner-membrane of the mitochondria by the action of CACT. Upon entry into the mitochondria matrix the carnitine ester is converted back to its acyl-CoA derivative by CPT-II. One cycle of mitochondrial β-oxidation results in the formation of 2(R),6-dimethylheptanoyl-CoA. This product is converted to its 2S-isomer by AMACR present in the mitochondria, enabling β-oxidation to proceed to completion forming acetyl-CoA.

5.1.4.2 Disorders of mitochondria function

There are five principal inborn errors of mitochondrial metabolism that have been reported to affect FA oxidation.

1- CPT deficiency- As described in section 5.1.2 the entry of LCFAs into the mitochondria and into β-oxidation is facilitated by three carnitine dependent proteins CPT-I, CPT-II and CACT. Deficiency of any of these proteins, therefore, results in reduced or blocked mitochondrial β-oxidation of LCFAs.

CPT-I deficiency is rare. There are two isoforms of CPT-I, which include a hepatic and a muscle form. Patients reported with CPT-I deficiency to date have only demonstrated a lack in the activity of the hepatic form (Olpin et al., 2001).

CPT-II deficiency is the most common cause of abnormal lipid metabolism in skeletal muscle and/or hepatic tissue (OMIM; 600650). There are two forms of CPT-II deficiency, the milder more common myopathic adult form and the severe neonatal hepatic form. The difference between the two forms has been studied at a molecular genetic level. The infant form has been found to demonstrate 10% residual CPT-II activity and impaired LCFA oxidation, whereas the adult form demonstrates a 20% residual CPT-II activity with no consequence on LCFA oxidation (Bonnefont et al., 1996).

CACT deficiency (OMIM: 212138) is caused by insertions (Huizing et al., 1997), deletions (Huizing et al., 1998; Ogawa et al., 2000) and mutations (Ogawa et al.,

2000) in the CACT gene. Seizures and episodes of coma may be provoked by fasting as CACT protein is responsible for the transfer of fatty acyl carnitines into the mitochondria, whereby mitochondrial oxidation of FAs provides the chief source of energy during prolonged fasting.

2- Acyl-CoA dehydrogenase deficiency – As discussed previously there are four enzymes that are responsible for the catalysis of the first step of each cycle of β-oxidation depending of the chain length of the FA. These are long, medium and short chain acyl-CoA dehydrogenase (LCAD, MCAD and SCAD) respectively. Clinical features include for both MCAD and LCAD are similar and include hypoglycemia and cardiomyopathy (Hale et al 1985). SCAD shows essentially two phenotypes, the neonatal form were patients show muscle weakness (Roe and Ding, 2001) and the middle-aged form where patients develop chronic myopathy (Turnbull et al., 1984).

3- 3-hydroxyacyl-CoA dehydrogenase deficiency- A deficiency of long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) leads to a build up of long-chain 3-hydroxy fatty acids, which are potentially toxic, whereby patients develop neuropathy and degenerative retinopathy (Tyni and Pihko, 1999).

4- Complete MTP deficiency - All three enzyme activities of this protein are deficient due to multiple mutations in either the α- or β-subunits. This disorder includes LCHAD deficiency and exhibits similar clinical features to LCHAD deficiency. However, complete MTP deficiency is generally a more severe disorder with earlier presentation and severe cardiac involvement (OMIM: 600890).

5- *Disorders of mitochondrial respiratory chain enzymes*– These disorders are caused by the loss or deletion of pieces of mitochondrial DNA (mtDNA), where different proportions of deleted mtDNA result in defects in subunits of respiratory enzyme complexes in various tissues. Pearson et al in 1979, described multiorgan disorders that were later found to be several diverse over-lapping syndromes caused by mutations of mtDNA (OMIM; 557000). Biochemical characteristics associated with this group of disorders referred to as Pearson's syndrome include mild lactic acidosis and reduced respiratory chain function in white cells and muscle biopsies.

5.1.4.3 Peroxisomal disorders

Three main categories of peroxisomal defects have been described to date.

Category A: The Zellweger syndrome (ZS) phenotype is caused by mutations in any of several different genes involved in peroxisomal biogenesis. The first description of a human disorder being linked to a loss of peroxisomal function was reported by Goldfischer et al (1973) who showed that peroxisomal matrix proteins were not properly compartmentalised in a group of patients diagnosed with Zellweger syndrome (OMIM: 214100).

Category B: Rhizomelic chondrodysplasia punctata (RCDP) was described later (Heymans et al., 1985), and was immediately linked to the peroxisome. Unlike the Zellweger spectrum of peroxisomal biogenesis disorders (PBDs) (category A), RCDP is genetically homogenous and only a small subset of peroxisomal enzymes are

mislocalised. Because of mutations in the gene encoding the PTS2 receptor located on the amino-terminus (N-terminus) of many peroxisomal enzymes and involved in peroxisomal import (Motley et al., 1994) the proteins are mislocalised to the cytosol. This form is referred to as type 1 RCDP (OMIM: 215100). Two other forms of RCDP have also been characterised, and are genetically distinct resulting from mutations in the genes for the peroxisomal enzymes, dihydroxyacetonephosphate-acyl transferase (DHAP-AT) (type 2 RCDP) (OMIM: 222765) and alkyl-dihydroxyacetonephosphate synthase (alkyl-DHAP synthase) (type 3 RCDP) (OMIM: 600121) which are involved in ether phospholipid synthesis.

Category C: The third category of peroxisomal disorders, result from single enzyme defects caused by specific mutation or loss of a single gene. These disorders are protein specific and include, X-linked adrenoleukodystrophy (XALD), short-chain acyl-CoA oxidase deficiency (SCOX-deficiency), D-bifunctional protein deficiency (D-BP deficiency), and α-methylacyl-CoA racemase deficiency (AMACR deficiency).

XALD (OMIM: 300100) is the most commonly diagnosed peroxisomal disorder. The condition is caused by a defect in an 80kDa peroxisomal membrane protein, adrenoleukodystrophy protein (ALDP). This protein belongs to the ATP binding cassettte (ABC) transporter family and is involved in the transport of VLCFAs and C26:0- CoA esters, across the peroxisomal membrane into the peroxisomal matrix (Mosser et al., 1994).

The deficiency of the SCOX enzyme (OMIM: 264470) causes VLCFA accumulation, whilst concentrations of pristanic acid and the bile acid intermediates, DHCA and THCA are normal.

D-BP deficiency (OMIM: 261515) was first discovered by Watkins et al., 1989). Two types of this disorder exist which include complete D-BP deficiency (Watkins et al., 1989; Van Grunsven et al., 1999b) and an isolated deficiency with a mutation in either of the two functional sites of the protein (D-3-hydroxyacyl-CoA dehydrogenase)(Van Grunsven et al., 1998) or D-BP enoyl-CoA hydratase (Van Grunsven et al., 1999a). Biochemically, VLCFA levels are abnormally high, and pristanic and phytanic acid concentrations are also elevated in all types of D-BP deficiency.

AMACR deficiency (OMIM: 604489) is associated with neurological disease in adult life caused by mutations in the gene encoding peroxisomal AMACR. Symptoms are relatively mild. Biochemically there is an accumulation of exclusively (R)-isomers of pristanic acid and the C27-bile acid intermediates DHCA and THCA in plasma of patients (Ferdinandusse et al., 2001).

5.2 Urinary vitamin E metabolites of patients with peroxisomal and mitochondrial disorders

5.2.1 Introduction

The side-chain shortened products of the tocopherols are recognised urinary metabolites, and include the α, β, δ and γ-homologues of carboxyethylhydroxy chromans (CEHCs) and carboxymethylbutylhydroxy chromans (CMBHCs) (chapter 1 section 1.6.2, figure 1.7). If the peroxisomes and/or the mitochondria are involved in the β-oxidation of the tocopherols, alterations in the concentration of these urinary metabolites might be expected in urine of patients with peroxisomal and/or mitochondrial disorders compared to those of healthy controls.

This section describes the investigation of urinary vitamin E metabolites of patients with peroxisomal disorders (n=5), mitochondrial disorders (n=4) and suspected peroxisomal or mitochondrial defects (n=5) compared to age and sex matched controls (n=17).

5.2.2 Experimental protocol

The collection of urine was conducted at either Great Ormond Street Hospital from patients with diagnosed or suspected peroxisomal or mitochondrial disorders, under

the care of Professor P Clayton and Dr S Rahman, or from King's College Hospital. Prior to analysis the urine samples were stored at –80°C within 12 hours of collection. The age and sex matched control urine samples were collected from subjects from Great Ormond Street Hospital, admitted to the hospital for non-metabolically related surgical procedures. This study was carried out with ethical approvel from the national standard NHS research ethics committee.

As it was not practical to collect 24 hour urine samples from each patient, spot urine samples were obtained and the metabolite concentrations were expressed per urine creatinine concentration.

The age and sex of the controls are shown in table 5.1, and brief details of the patients are shown in table 5.2. The age range of the patients is from 0.7 to 470 weeks and for the controls from 10 to 489 weeks. Age match controls less than 10 weeks of age could not be recruited. Unless otherwise stated only one sample was collected from each patient.

5.2.3 Analysis of results

The urinary vitamin E metabolite concentrations for the control subjects and patients with peroxisomal and mitochondrial disorders were analysed. The metabolites measured include α, β, δ and γ-homologues of CEHC and CMBHC and concentrations are expressed in nmoles per mmol creatinine.

CONTROL	SEX	AGE (weeks)
C1	m	10
C2	m	12
C3	m	14
C4	f	22
C5	f	24
C6	m	32
C7	f	32
C8	f	52
C9	m	56
C10	f	121
C11	m	128
C12	f	180
C13	m	301
C14	f	328
C15	m	423
C16	m	459
C17	f	489

Table 5.1; The age and sex of the control subjects

	PATIENT	SEX	AGE (weeks)	DIAGNOSIS
Peroxisomal disorder	P1	m	0.7	Peroxisomal biogenesis defect; Zellweger syndrome
	P2	m	5.4	Peroxisomal biogenesis defect; Zellweger syndrome
	P3	m	470	Peroxisomal biogenesis defect; mild, mosaic
	P4	m	52	Peroxisome D-BP deficiency; 3-hydroxyacyl-CoA dehydrogenase defective
	P5	f	328	Peroxisome D-BP deficiency; mild, uncharacterised
Mitochondrial disorder	M1	f	382	Pearson's syndrome (mitochondrial respiratory chain defect)
	M2a)	m	12	Pearson's-like syndrome
	M2b)	m	14	(follow-up sample from patient M2a))
	M3	m	86	LCHAD deficiency
Suspected peroxisomal/ mitochondrial disorders	S1	f	136	mitochondrial defect
	S2	m	5	peroxisomal/mitochondrial defect?
	S3	m	253	mitochondrial – not respiratory chain defect
	S4	f	33	mitochondrial defect
	S5	f	25	mitochondrial defect

Abbreviations;
D-BP = D-bifunctional protein
LCHAD = Long-chain hydroxyacyl-CoA dehydrogenase

Table 5.2; Details of patients with peroxisomal and mitochondrial disorders

5.2.3.1 Control subjects

The individual concentrations and means of the urinary metabolites in the 17 control subjects are given in table 5.3 and the concentrations plotted against age are shown in figures 5.7 and 5.8. It can be seen that the urinary concentrations of α- and γ-CEHC (figure 5.7) and α and γ-CMBHC (figure 5.8) showed no consistent correlation with age. α-CEHC was present in the urine of all control subjects with concentrations between the range of 118 to 9,450 nmol/mmol creatinine. The concentrations of α-CMBHC were within the range of undetectable to 148nmole/mmole creatinine, where five of the seventeen subjects exhibit no detectable α-CMBHC. β-CEHC was present in all the subjects ranging from 10 to 1,639 nmol/mmol creatinine, whilst the concentrations of β-CMBHC were within the range of undetectable to 126nmole/mmole creatinine, with none detected in six subjects. δ-CEHC was present in all subjects with concentrations ranging from 6 to 892nmol/mmol creatinine. δ-CMBHC was absent in five subjects, with a range from undetectable to 114nmole/mmole creatinine. γ-CEHC was present in the urine of all the control subjects, with concentrations within the range of 60 to 4,351nmol/mmol creatinine. The concentrations of γ-CMBHC were within the range of undetectable to 178nmol/mmol creatinine, with six of the seventeen subjects showed undetectable levels. It is evident that the longer chain-shortened metabolites, the CMBHCs, are often undetectable in the urine of the control subjects. This tends to occur in an individual subject for more than one homologue of CMBHC, for example, the control subjects C7 and C11 showed the absence of α, β, δ and γ-CMBHC, C9 showed the

METABOLITE	Control subject																	mean (range)
	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	
α-CEHC	1568	194	520	3408	2929	570	118	344	126	9450	150	2390	172	145	278	484	6739	1740.3 (118-9450)
α-CMBHC	100	18	60	131	76	148	0	44	0	20	0	0	0	48	16	53	16	42.9 (0-148)
β-CEHC	10	30	10	154	71	122	188	411	55	123	100	1639	184	23	80	269	72	208.3 (10-1639)
β-CMBHC	20	9	20	0	0	126	0	6	0	22	0	85	0	46	33	26	24	24.5 (0-126)
δ-CEHC	265	36	400	892	735	187	6	67	116	53	713	766	55	13	115	122	63	239.1 (6-892)
δ-CMBHC	35	3	30	62	71	104	0	0	12	3	0	114	0	17	29	0	23	29.6 (0-114)
γ-CEHC	225	570	1800	3492	3271	1891	60	500	618	775	1123	4351	514	214	1181	1347	509	1320.1 (60-4351)
γ-CMBHC	25	3	0	0	0	178	0	17	0	12	0	84	48	92	102	93	36	40.6 (0-178)

Table 5.3; Concentrations of vitamin E metabolites in nmol/mmol creatinine for control subjects

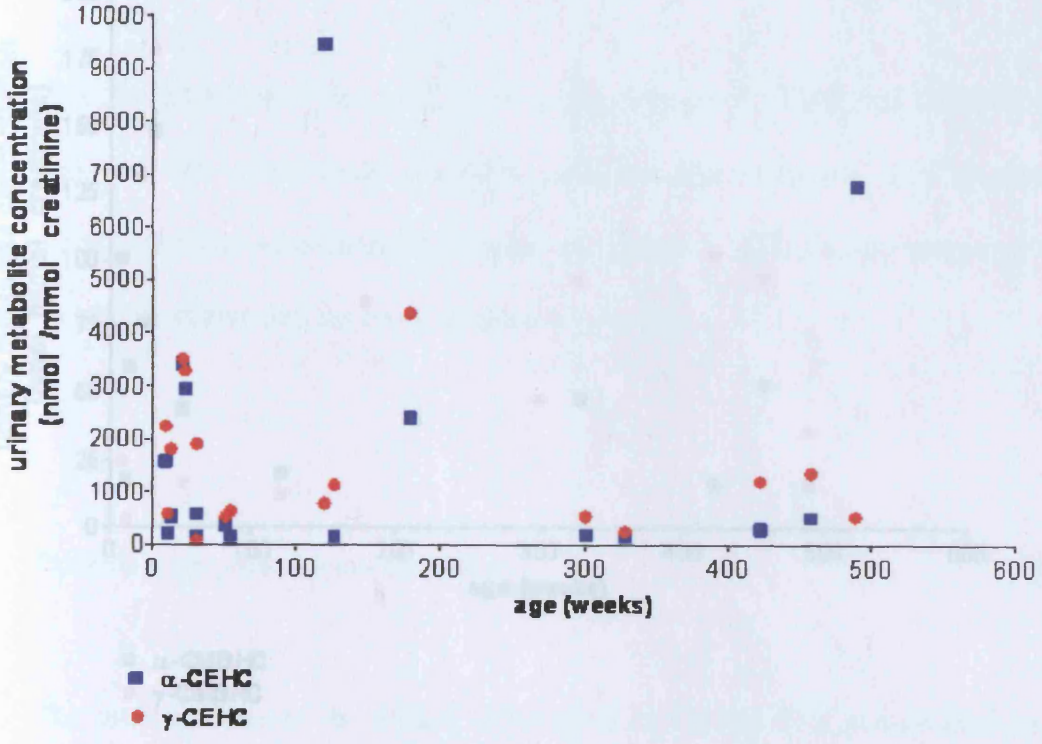


Figure 5.7; α-CEHC and γ-CEHC concentrations in urine of controls plotted against their age in weeks

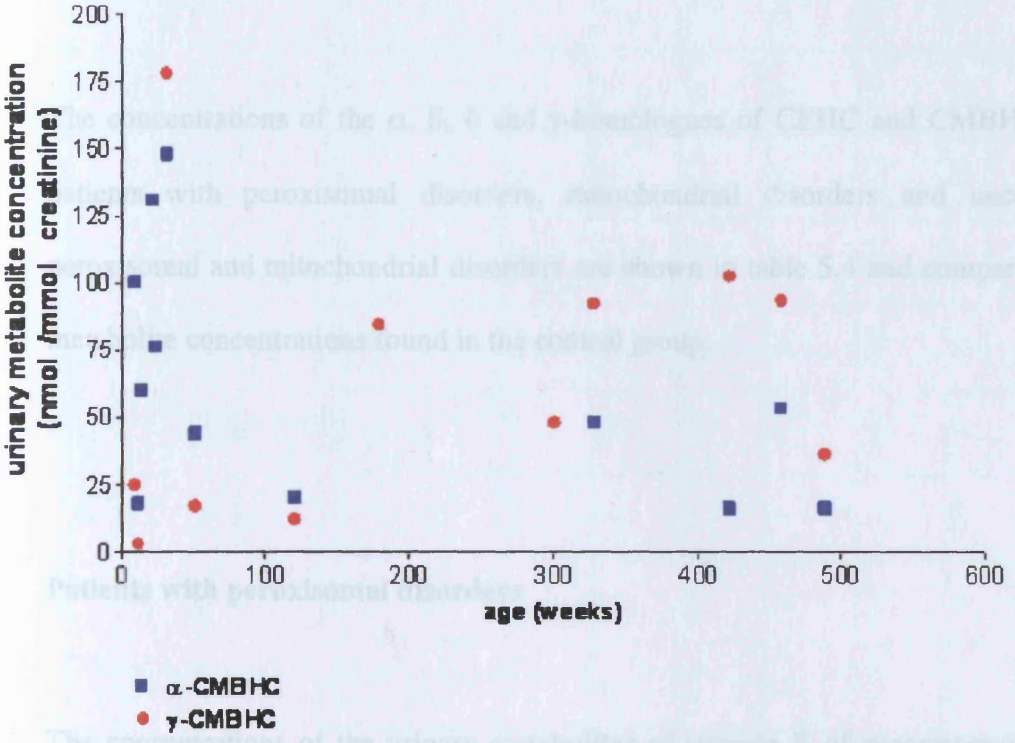


Figure 5.8; α-CMBHC and γ-CMBHC concentrations in urine of controls plotted against their age in weeks

absence of α, β, and γ-CMBHC and subject C13 showed the absence of α, β and δ-CMBHC.

5.2.3.2 Patients with peroxisomal and mitochondrial disorders

The concentrations of the α, β, δ and γ-homologues of CEHC and CMBHC of the patients with peroxisomal disorders, mitochondrial disorders and unconfirmed peroxisomal and mitochondrial disorders are shown in table 5.4 and compared to the metabolite concentrations found in the control group.

Patients with peroxisomal disorders

The concentrations of the urinary metabolites of vitamin E of peroxisomal patients demonstrated no consistent trends. P3 and P4 displayed concentrations of urinary vitamin E metabolites within the control ranges. For P1, only β- and δ-CEHC was detected. P2 had a urinary metabolite profile similar to that of C7 and C11, demonstrating a lack of CMBHCs and the concentration of α-CEHC (15nmol/mmol creatinine) was lower than the lower limit of the control range (110nmol/mmol creatinine). For P5, the concentration of α-CMBHC (515nmol/mmol creatinine) was higher than the maximum of the control range (148nmol/mmol creatinine).

METABOLITE	Patients														Control mean (range)
	P1	P2	P3	P4	P5	M1	M2 a)	M2 b)	M3	S1	S2	S3	S4	S5	
α-CEHC	0	15	181	270	676	388	0	1031	3727	1360	477	157	1003	429	1740.3 (118-9450)
α-CMBHC	0	0	71	0	515	23	0	86	302	21	65	18	0	39	42.9 (0-148)
β-CEHC	25	167	254	130	131	975	594	419	512	242	42	13	52	29	208.3 (10-1639)
β-CMBHC	0	0	42	55	24	0	0	163	37	47	50	11	31	18	24.5 (0-126)
δ-CEHC	50	15	40	50	108	23	141	913	132	651	477	21	0	318	239.1 (6-892)
δ-CMBHC	0	0	0	0	27	0	0	238	0	0	69	9	33	43	29.6 (0-114)
γ-CEHC	0	70	163	355	498	118	0	5663	693	2334	2023	175	3106	2675	1320.1 (60-4351)
γ-CMBHC	0	0	71	0	97	0	459	6	20	23	12	21	0	18	40.6 (0-178)

Table 5.4; Urinary vitamin E metabolite concentrations in nmol/mmol creatinine of patients with peroxisomal (P), mitochondrial (M) or suspected peroxisomal or mitochondrial disorders (S).

Patients with mitochondrial disorders

Patient M1 demonstrated normal urinary concentrations of all vitamin E metabolites analysed. For patient M2 urine sample (a) (taken at 12 weeks of age), no α-CEHC or γ-CEHC were detected, and the concentration of γ-CMBHC (459nmol/mmol creatinine) was greater than the maximum of the control range (178nmol/mmol creatinine). The metabolite concentrations for patient M2 sample (b) (taken at 14 weeks of age) demonstrated β- and δ-CMBHC and δ- and γ-CEHC concentrations elevated above the control ranges. For patient M3 the concentration of α-CMBHC was higher (302nmol/mmol creatinine) than the maximum of the control range (148nmol/mmol creatinine).

Patients with suspected peroxisomal or mitochondrial disorders

The concentrations of urinary metabolites of vitamin E of patients S1-S5 were within the normal range except for S4 (refer to table 5.4) where δ-CEHC was not detected.

Metabolite concentrations in patients and controls as a function of age

Figures 5.9 a and b, show the concentrations (nmol/mmol creatinine) of α-CEHC and γ-CEHC respectively, for each control subject and patient as a function of age (weeks). It can be seen that in general the urinary concentrations of α- and γ-CEHC of

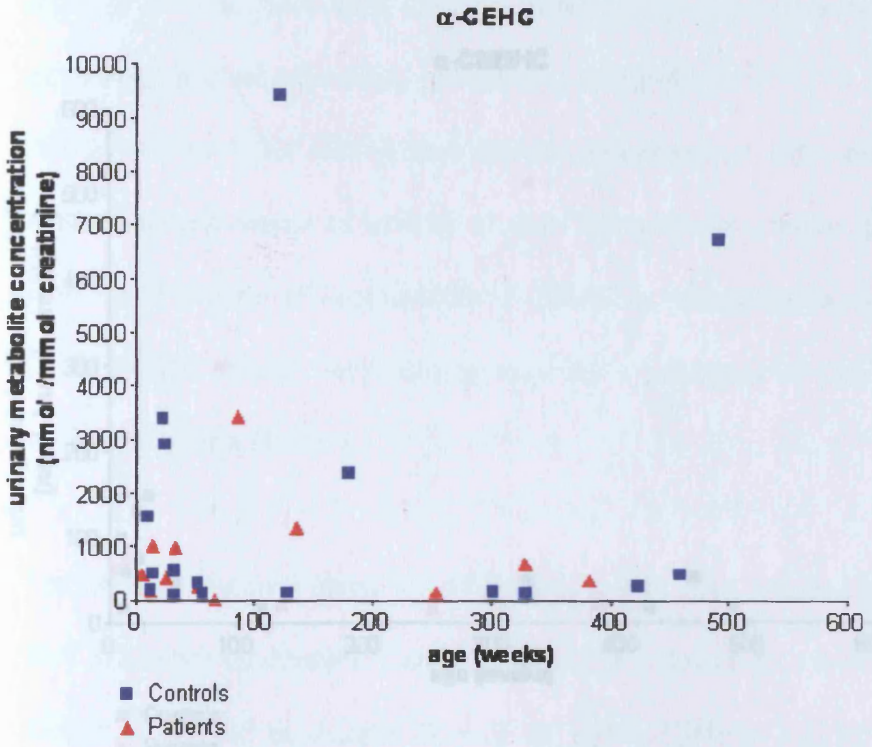


Figure 5.9 a); α-CEHC concentrations in urine of controls and patients plotted against their age in weeks

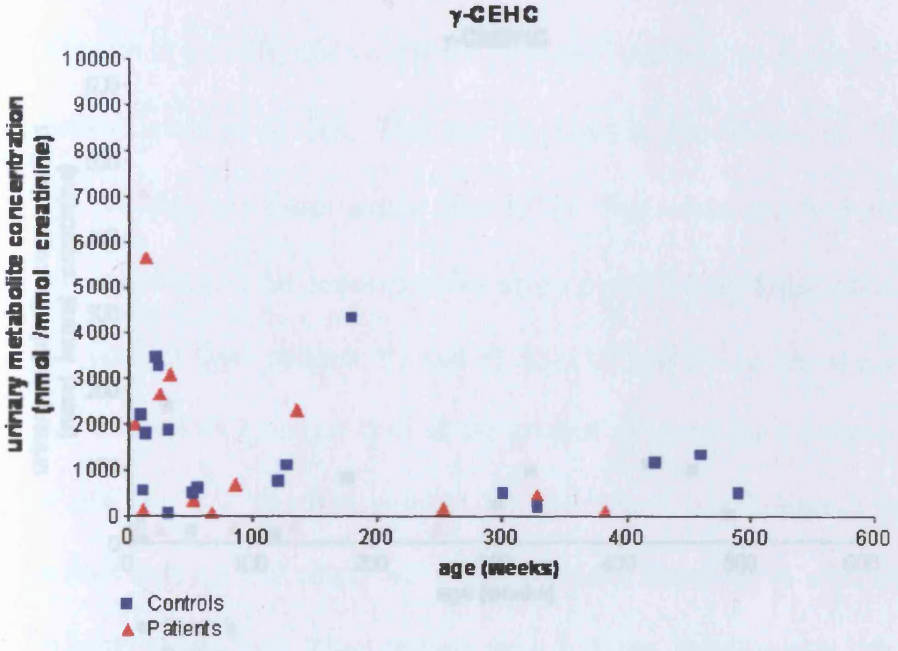


Figure 5.9 b); γ-CEHC concentrations in urine of controls and patients plotted against their age in weeks

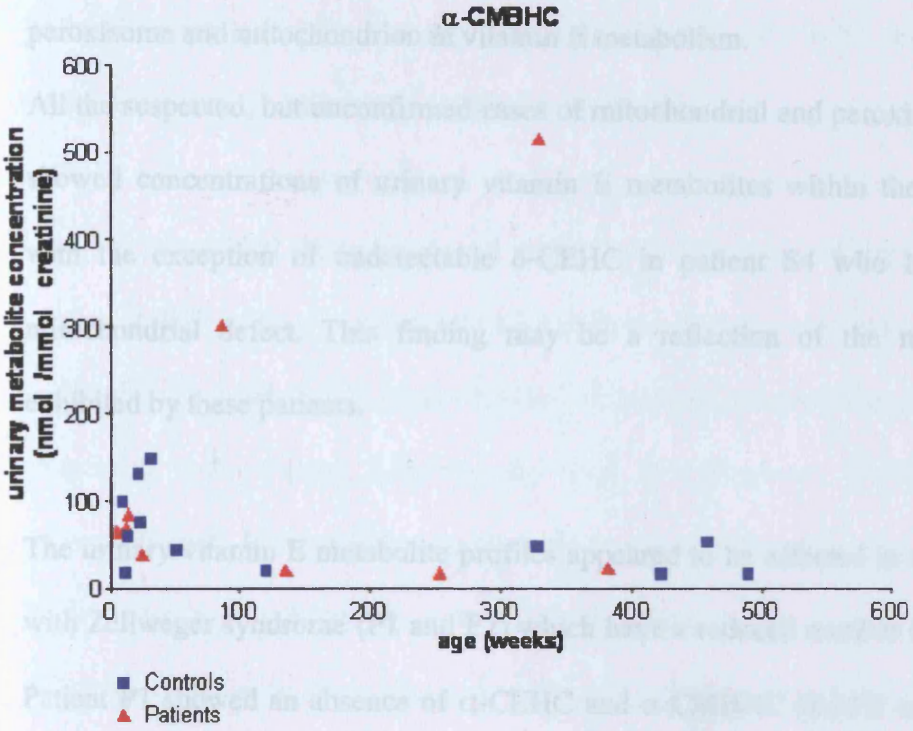


Figure 5.10 a); α-CMBHC concentrations in urine of controls and patients plotted against their age in weeks

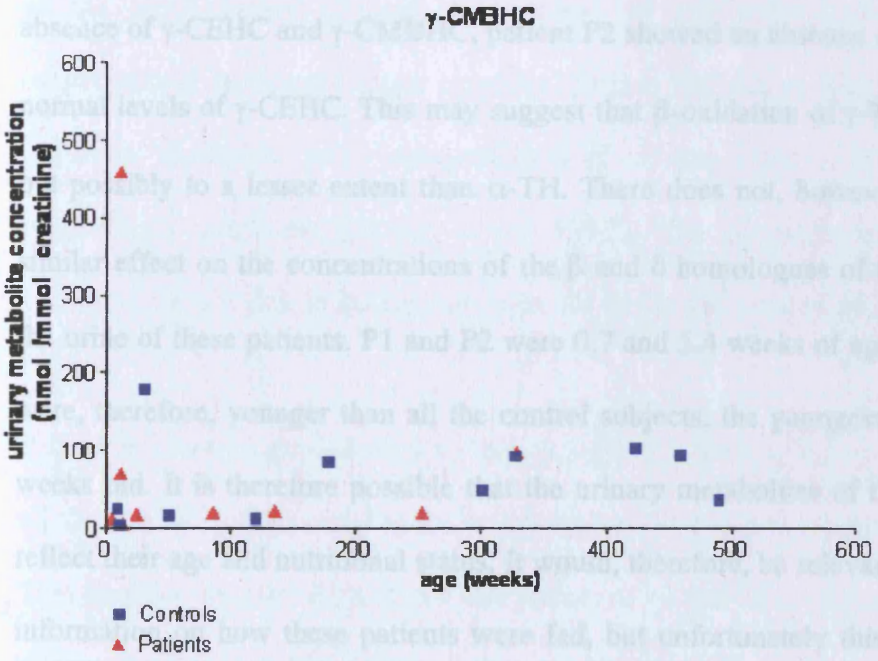


Figure 5.10 b); γ-CMBHC concentrations in urine of controls and patients plotted against their age in weeks

difficult to come to a firm conclusion regarding the precise involvement of the peroxisome and mitochondrion in vitamin E metabolism.

All the suspected, but unconfirmed cases of mitochondrial and peroxisomal disorders, showed concentrations of urinary vitamin E metabolites within the control ranges with the exception of undetectable δ-CEHC in patient S4 who had a suspected mitochondrial defect. This finding may be a reflection of the mild phenotypes exhibited by these patients.

The urinary vitamin E metabolite profiles appeared to be affected in the two patients with Zellweger syndrome (P1 and P2) which have a reduced number of peroxisomes. Patient P1 showed an absence of α-CEHC and α-CMBHC and P2 an absence of α-CMBHC with a greatly reduced α-CEHC concentration (approximately ten fold lower than the lower limit of the control range), suggesting that the peroxisomes may be involved in the chain-shortening of α-TH. Although, patient P1 also showed an absence of γ-CEHC and γ-CMBHC, patient P2 showed an absence of γ-CMBHC with normal levels of γ-CEHC. This may suggest that β-oxidation of γ-TH is also effected but possibly to a lesser extent than α-TH. There does not, however, appear to be a similar effect on the concentrations of the β and δ homologues of CEHC detected in the urine of these patients. P1 and P2 were 0.7 and 5.4 weeks of age respectively and were, therefore, younger than all the control subjects, the youngest of which was 10 weeks old. It is therefore possible that the urinary metabolites of these patients may reflect their age and nutritional status. It would, therefore, be relevant to have detailed information on how these patients were fed, but unfortunately this information was not available.

Patient P3 was diagnosed with a mild peroxisome biogenesis defect (PBD), with a reduced number of peroxisomes. The concentrations of all the urinary vitamin E metabolites in these patients fell within the control range.

Patients P4 and P5 were diagnosed with D-BP deficiency which consists of three groups of deficiencies; 1- complete D-BP deficiency; 2- isolated enoyl-CoA hydratase deficiency; 3- isolated 3-hydroxyacyl-CoA dehydrogenase deficiency. Patient P4 was diagnosed with isolated 3-hydroxyacyl-CoA dehydrogenase deficiency, and had urinary vitamin E metabolite concentrations which fell within the control ranges. Patient P5 was also diagnosed with D-BP deficiency, but the specific defect of the D-BP protein has yet to be determined. All urinary vitamin E metabolite concentrations fell within the control range, except for the concentration of α-CMBHC which was more than three fold greater than the upper limit of the control range.

In the mitochondrial disorder group, two of the patients had defects of the mitochondrial respiratory chain enzymes. Patient M1 was diagnosed with Pearson's syndrome exhibiting some characteristics of another mtDNA deletion disorder - Kearn-Sayre syndrome (Morris et al., 1997). The concentrations of the urinary vitamin E metabolites in the patients were all within the control ranges.

Patient M2 was diagnosed with a Pearson's-like syndrome. This syndrome has many of the clinical features of Pearson's syndrome but has normal mitochondrial DNA. Two samples were analysed from this patient at 12 and 14 weeks of age. There were differences in the concentrations of the urinary vitamin E metabolites between the two samples which could be largely explained by treatment during this period. Patients

with Pearson's-like syndrome exhibit pancreatic insufficiency and during the period in which the two samples were taken, the patient was treated with pancreatic enzyme replacement and also received Intralipid intravenously to maintain energy, lipid and nutrient balance. Intralipid contains soya oil where the predominant form of vitamin E is γ-TH, with high δ-TH and low concentrations of α- and β-TH. In addition the patient was also administered Vitlipid in order to increase vitamin E supplementation levels. Vitlipid contains synthetic *all-rac*-α-TH. In response to treatment there was significant clinical improvement and plasma vitamin E concentrations were normalised. During the treatment period all the vitamin E metabolites became detectable and some of them (δ-, γ-CEHC and β- and δ-CMBHC) increased above the upper end of the control range. Unfortunately plasma vitamin E concentration data was not available for all the subjects.

The patient with LCHAD deficiency (M3) had concentrations of vitamin E metabolites which fell within the control range except for an increased α-CMBHC concentration.

In summary, there was some evidence from the two patients with Zellweger syndrome that the peroxisome may be involved in chain shortening of the tocopherols. In general, however, the changes in urinary metabolite concentrations observed in these patients with mitochondrial and peroxisomal abnormalities were not consistent and not as severe as might have been expected. It would appear that sufficient enzyme activity was present to preserve peroxisomal and mitochondrial pathways in these patients who have survived, as complete elimination of such pathways are likely to be incompatible with life.

Chapter 6

In vitro investigation of the cellular localisation of tocopherol metabolism

6.1 Introduction

The aim of this study was to investigate the role of peroxisomes and mitochondria in vitamin E metabolism comparing the metabolism of α-TH and γ-TH in cultures of human skin fibroblasts and human hepatoblastoma (HepG2) cells supplemented with these tocopherols.

Two different approaches were attempted. Firstly, skin fibroblasts from patients with specific peroxisomal and mitochondrial defects were investigated and secondly, mitochondrial and peroxisomal functions were manipulated in HepG2 cells.

6.1.1 Manipulation of mitochondrial fatty acid oxidation

It was decided to use etomoxir (sodium 2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate) as a mitochondrial β-oxidation inhibitor for the manipulation of mitochondrial FA oxidation in this study. Etomoxir is an inhibitor of CPT-I, which is the first committed step of mitochondrial FA oxidation, and is the rate-limiting step. Many studies employ CPT-I inhibitors as a control of overall FA oxidation (Barnett et al., 1992; Suzuki et al., 1991).

Etomoxir was chosen as a mitochondrial inhibitor in this investigation rather than other CPT-I inhibitors, due to its ready availability and experience of its use in our department.

6.1.2 Manipulation of peroxisomal fatty acid oxidation

Studies involving the regulation of peroxisomal FA β-oxidation, have mainly investigated the effect of peroxisome proliferation, and a number of reliable peroxisome proliferators are available. It was decided to use perfluorooctane sulphonate (PFOS) as a peroxisome proliferator for the manipulation of peroxisomal FA oxidation in this study. PFOS is commercially available and relatively inexpensive, and unlike most other peroxisome proliferators does not affect mitochondrial bioenergetics and biogenesis.

PFOS belongs to a family of perfluorinated alkyls, which includes the well studied perfluooctanoic acid (PFOA). PFOA is a stable and non-oxidisable FA analog that causes peroxisome proliferation both in vivo (Ikeda et al., 1985) and in vitro (Intrasuksri and Feller, 1991), independent of mitochondrial β-oxidation. Intrasuksri et al (1998) showed that the stimulatory effects of PFOA are due to the stimulation of acyl-CoA oxidase (ACO) activity in hepatocytes. It was suggested that the increase in ACO activity was due to de novo enzyme synthesis, as cyclohexamide (an inhibitor of protein synthesis), caused complete blocking of ACO activity. It appears that PFOA like other non-β-oxidisable FAs and xenobiotics, interacts directly to up-regulate expression of the peroxisome proliferator response genes through the activation of PPAR-α. PPAR-α is a ligand dependent transcription factor, whereby any elevation in enzyme activity mediated through this receptor would increase protein synthesis. PFOS is also able to induce peroxisomal proliferation (Berge et al., 1989; Sohlenius et al., 1993) and in contrast to PFOA, contains a sulphur substitution at the β-carbon. Many studies have reported that compounds that cause peroxisome proliferation in

rodents also interfere with mitochondrial bioenergetics and biogenesis. This led to the suggestion that the proliferation of the peroxisome and the mitochondria may be related. However, PFOS unlike PFOA and other peroxisome proliferators, causes peroxisome proliferation without affecting the mitochondria, and this therefore suggests that peroxisome proliferation can be independent of the effects on mitochondrial function (Berthiaume and Wallace, 2002). Berthiaume and Wallace (2002), showed that PFOS and PFOA have similar potencies for peroxisome proliferation, however, PFOS did not cause the characteristic hepatomegaly observed by PFOA, suggesting some differences in their mechanism of action (Berthiaume and Wallace, 2002).

6.2 Metabolism of vitamin E in human skin fibroblasts and HepG2 cells in culture

6.2.1 Cell culture techniques

A normal fibroblast cell line was obtained as a growing cell culture, which had been established from a skin biopsy, from a metabolically normal patient, by the Enzyme Laboratory at Great Ormond Street Hospital. Fibroblasts were grown as mono-layers in tissue culture flasks, which had a surface area of 25cm^2 and contained 5ml Hams F10 growth medium. The Hams F10 growth medium contained 25mM HEPES and

1mM L-glutamine, and was supplemented with 12% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotic solution (1mg/ml). Once prepared, the medium was stored for up to one month at 4^0C.

The HepG2 cell line was supplied by the European Collection of Cell cultures (ECACC-85011430) as a frozen aliquot. Upon reconstitution of the cell line, cultures were grown in Dulbecco's modified eagle medium (D-MEM (MOD)), with 10%FBS and 1% penicillin/streptomycin. Cultures were initially left to grow to confluency for approximately 6 days.

Cell cultures were incubated at 37^0C with 5% (v/v) CO_2 in an incubator. The growth medium was replaced twice a week and the cells in each flask were sub-cultured, harvested or stored in liquid nitrogen as required, depending on their confluency, which was validated by phase contrast microscopy.

Materials

All cell culture procedures were carried out under sterile conditions. Disposable sterile plastic-ware was obtained from Falcon. Culture medium, FBS (EC approved origin), phosphate buffered saline (PBS) and trypsin solution (0.25%) were supplied by Gibco-life technologies, and penicillin/streptomycin antibiotic solution (1mg/ml) supplied by Sigma Chemicals. Trypsin buffer was made up in distilled water and consisted of 0.1M sodium chloride and 10M sodium citrate. All culture solutions were sterilised by filtering through a Millex 0.22μm syringe filter before use. Glassware was autoclaved at 125°C for 30 mins.

Trypan blue stain solution (4%) and 4,6-Diamidino-2-phenylindole (DAPI) stain were purchased from Sigma Chemicals.

(+)-γ-Tocopherol (RRR-γ-TH)(T-1782) and (±)-α-tocopherol (all-rac-α-TH)(T-3251) were purchased from Sigma Chemicals and made up to 500mM stock solutions in acetone. Perflurooctane sulphonic acid (PFOS) was supplied by Fluka Chemicals. Etomoxir was a gift from Dr Simon Eaton, Surgery Unit, The Institute of Child Health, London. Etomoxir was made up to a 1mM solution in ultrapure water.

Cell culture techniques

Cell culture techniques, including 1)- subculturing cell cultures, 2)- harvesting and storage of cultures, 3)- harvesting cells for analysis and 4)- reconstituting cells from storage in liquid nitrogen, were carried out using standard protocols as detailed by the Enzyme Laboratory, Great Ormond Street Hospital. Briefly, cells were released from the plastic flasks by trypsinisation using sterile trypsin solution (0.25%) for 5-10 minutes at 37°C. Cells harvested for storage in liquid nitrogen were suspended in ice-cold growth medium containing 10% (v/v) dimethyl sulphoxide (DMSO). Cells harvested for analysis were washed then suspended in PBS and stored at –20°C.

Cell Viability and screening for mycoplasma contamination-

The cell viability of the cultures was estimated using the 'trypan blue viability test' and expressed as a percentage of the viable cells compared to total number of cells. Cell lines were screened for mycoplasma contamination by in situ DNA fluorescence, using DAPI (4,6-diamidino-2-phenylindole) staining. None of the cultures examined exhibited the presence of mycoplasma.

6.2.2 α-Tocopherol supplementation of cell cultures

6.2.2.1 Supplementation of human skin fibroblast cultures with α-tocopherol

The first experiments involved the supplementation of human skin fibroblasts with all-rac-α-TH. Metabolism of the supplemented TH was monitored by the analysis of metabolites in the spent culture medium at the end of the incubation period.

Preparation of supplemented growth medium

The supplementation of the growth medium was based on the protocol used by Tran and Chan (1992) and Young et al (2001) involving the dispersion of TH in FBS before addition to the medium;

1- FBS was allowed to equilibrate at 37°C in a water-bath and 500mM α-TH at room temperature for approximately 20 minutes.

2- 80 μl of 500mM all-rac-α-TH (in acetone) was added to 30 ml FBS. N_2 was briefly blown over the surface of the FBS to remove the acetone. The mixture was placed in a 37°C water-bath for 20 mins and then rotated at room temperature for 2 hrs. The mixture was then sterilized by passing through a 0.22μ Millex syringe filter.

3- Determination of α-TH concentration in supplemented FBS samples

Three 50μl aliquots of the filtered FBS were removed and the α-TH extracted by solvent extraction and its concentration determined by normal phase high performance liquid chromatography (HPLC) with fluorometric detection (Metcalfe et al., 1989) by a modification of the method Buttriss and Diplock, (1983) as follows;

i) 1ml methanol was added to 50μl of the supplemented FBS, vortexed and then 1ml hexane was added. The mixture was vortexed and centrifuged at 2,500g for 5 minutes. The upper hexane phase containing the extracted TH was removed.

ii) α-TH concentration of the FBS was determined by injecting 20μl of the upper hexane phase on to a normal phase HPLC column. The HPLC system consisted of a spectra-Physics SP8770 isocratic pump (pressure at 28-30kPsi, flow rate of 1ml/min), Shimadzu Fluorescence HPLC monitor RF-535 (excitation wavelength 295nm, and emission wavelength 320nm), Shimadzu Chromatopac C-R8A integrator and an APEX silica 3μ 1070428 chromatography column with a guard column.

iii) α-TH concentration was calculated from a standard curve using GraphPad Prism and the concentrations of α-TH in FBS calculated manually.

4- The amount of TH supplemented FBS to be added to the medium to give the required α-TH concentration was calculated.

5- The remaining required ingredients of the growth medium were made up prior to addition of the TH supplemented FBS, as follows; Hams F10 growth medium, unsupplemented FBS (to make up total final FBS concentration to 10%) and antibiotic solution (penicillin/streptomycin, at 1%) were mixed together, then sterilized by passing through a 0.22μ "Millex" syringe filter.

6- The sterilized (filtered) TH supplemented FBS was added to the sterilized medium made in step 5, and was mixed by rotation for 10-15 mins.

7 The final concentration of α-TH in the medium was measured in 100μl aliquots of medium, using normal phase HPLC with fluorimetric detection as in step 2.

8- The medium was placed in a 37°C water-bath for 20 minutes, before feeding to the cell cultures.

Analysis of spent culture media and cells for α-tocopherol and α-tocopherol metabolites

Cell cultures were incubated with the appropriate medium and at the end of the incubation period, the medium was removed from each culture flask and stored at -20°C until analysis. Cells were harvested by trypsinisation, and stored in trypsin buffer solution at –20°C until analysis.

Extraction of tocopherol from the spent media and cell suspension

Extraction of α–TH from both the spent media, and cell suspensions were carried out using the solvent extraction method and normal phase HPLC with fluorescence detection as described above for supplemented FBS, using 50μl of the cell suspension and 100μl of media respectively.

Protein determination of cell cultures

Protein determination was based on the bicinchoninic acid (BCA) method of Smith et al (1985).

Extraction of tocopherol metabolites from media

Initially vitamin E metabolites were extracted from the spent medium using solid phase extraction with a C4 cartridge supplied by Jones Chromatography similar to the method developed for the extraction of urinary metabolites (chapter 2). The deconjugation and the second solid phase extraction step were not carried out, as unlike human urine, TH metabolites secreted by HepG2 cells appear to be unconjugated (Parker and Swanson, 2000). This was later confirmed by Dr Mills in our laboratory using tandem mass spectrometry. Preparation for GC-MS analysis was also performed as described for the urinary metabolites. It was, however, found that the extraction from the media was incomplete using this method, since in addition to the lack of peaks for the metabolites, no peak was detected for the internal standard (d9-α-CEHC), which was added to the sample prior to the extraction.

Parker and Swanson (2000) studied the metabolism of γ-TH in HepG2 cells in culture and described a method for the extraction and measurement of TH metabolites from the medium. The procedure involved extracting the metabolites into methyl tertiary-butyl ether (MTBE). The MTBE phase was then evaporated to dryness and derivatised for gas chromatography-mass spectrometry (GC-MS) analysis. Using this method, Parker and his group were able to detect both γ-CEHC and the longer chain metabolite, γ-CMBHC, in the growth medium.

Parker and Swanson's protocol adapted for the present study was as follows.
The samples of growth media, 5ml in volume, were spiked with internal standard, d9-α-CEHC, to give a final concentration of 1μM and acidified to pH1.5. The samples were vortexed with 5ml MTBE for 20 seconds and then centrifuged at 4,000g for 10 minutes. The upper phase, containing the TH was removed, and the extraction step was repeated on the remaining lower phase. The upper phases were combined and evaporated to dryness. The samples were then derivatised with 150 μl acetonitrile/BSTFA (2:1), at 60°C for 1 hour. Derivatised samples were evaporated to approximately one third their volume and run on GC-MS using the same parameters as for the urinary vitamin E metabolites (see chapter 2).

Results of the preliminary experiment

The solvent extraction method used to extract vitamin E metabolites from the spent medium from skin fibroblasts provided good detection of the internal standard, d9-α-CEHC and α-TH, but no α-TH metabolites were detected (figure 6.1).

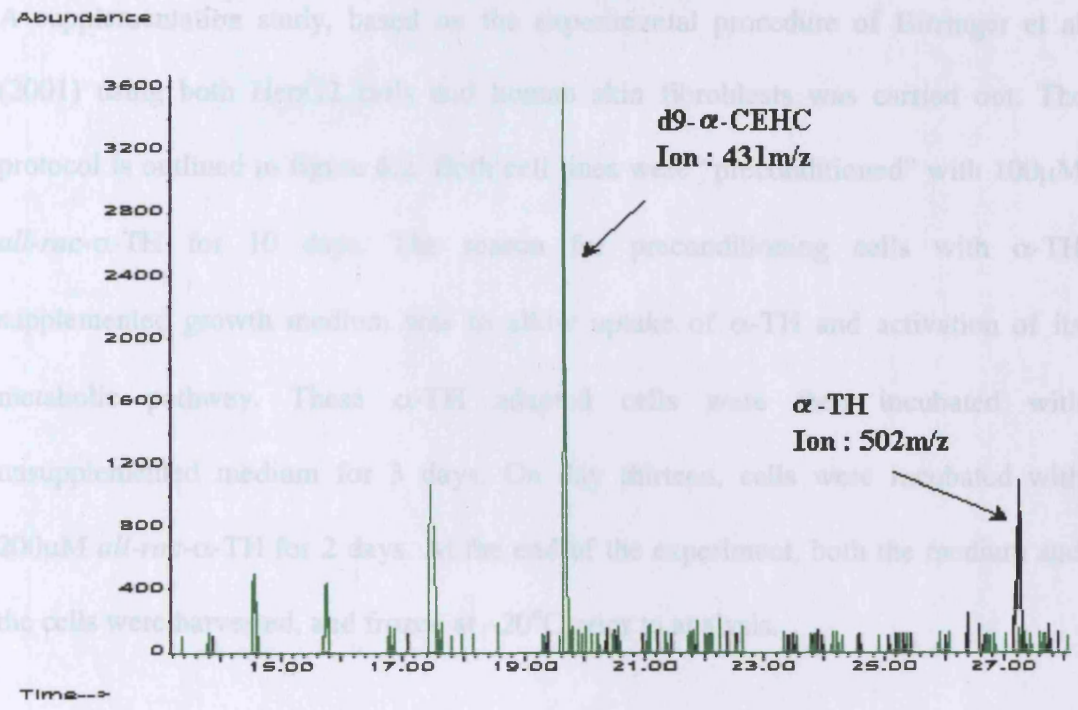


Figure 6.1; Selected ion chromatogram showing the internal standard, d9-α-CEHC, and α-TH in harvested culture medium from skin fibroblasts. No vitamin E metabolites were detected.

6.2.2.2 Preconditioning cultured HepG2 and human skin fibroblast cell lines with α-tocopherol

A supplementation study, based on the experimental procedure of Birringer et al (2001) using both HepG2 cells and human skin fibroblasts was carried out. The protocol is outlined in figure 6.2. Both cell lines were "preconditioned" with 100μM *all-rac*-α-TH for 10 days. The reason for preconditioning cells with α-TH supplemented growth medium was to allow uptake of α-TH and activation of its metabolic pathway. These α-TH adapted cells were then incubated with unsupplemented medium for 3 days. On day thirteen, cells were incubated with 200μM *all-rac*-α-TH for 2 days. At the end of the experiment, both the medium and the cells were harvested, and frozen at –20°C prior to analysis.

Results of supplementation study with preconditioning of HepG2 cells and human skin fibroblast cells

Measurement of α-TH in harvested cells and media

Triplicate flasks of skin fibroblasts and HepG2 cells were "preconditioned" and supplemented with α-TH and compared to blanks without added α-TH. The amounts (μmoles) of α-TH recovered in the medium and in the harvested cells were examined for all triplicate flasks of both cell lines throughout the course of the feeding protocol, and the mean results are shown in figure 6.3. The triplicate flasks of both cell lines

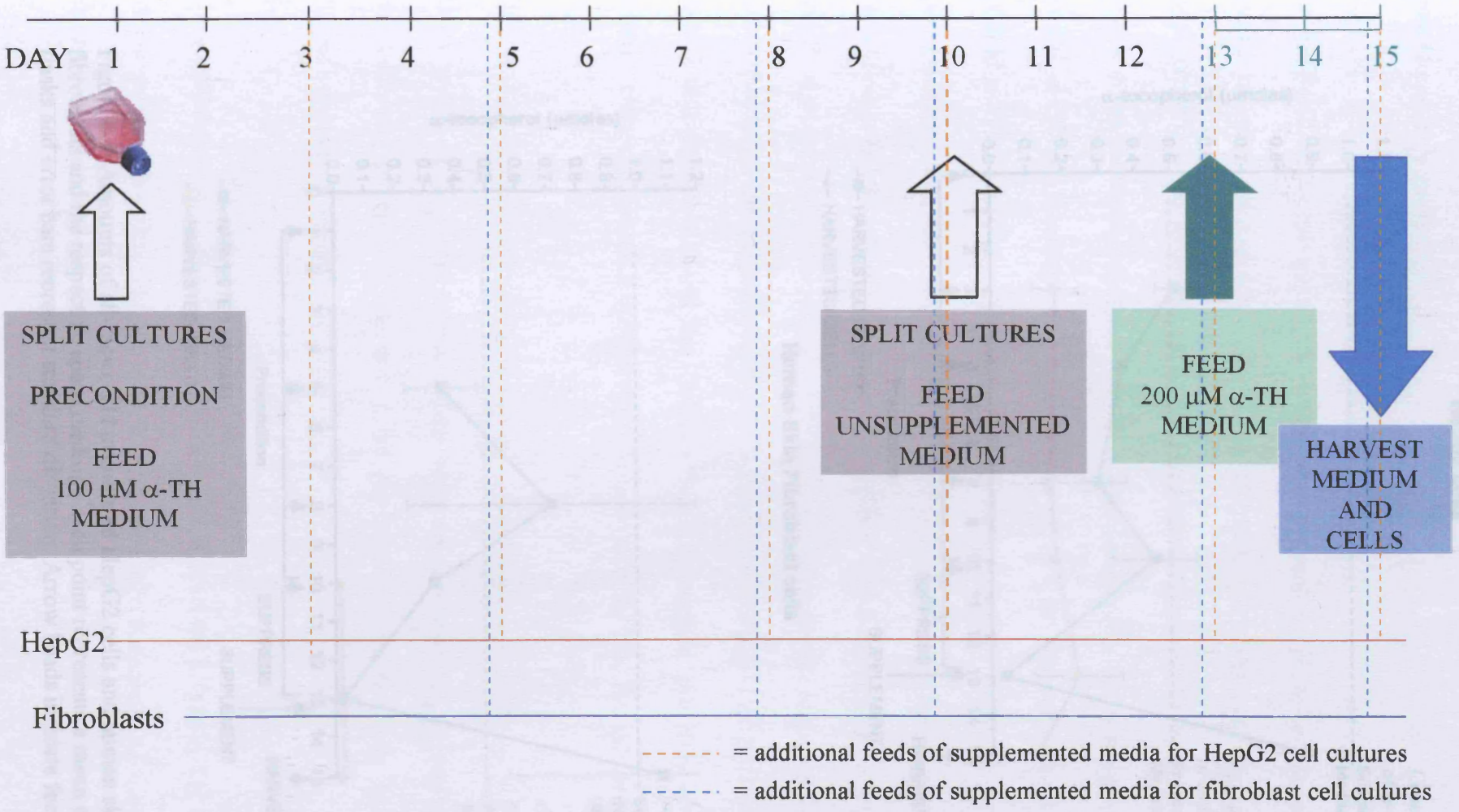


Figure 6.2; "Preconditioning" and feeding programme for HepG2 and human skin fibroblast cultures with α-TH supplemented medium

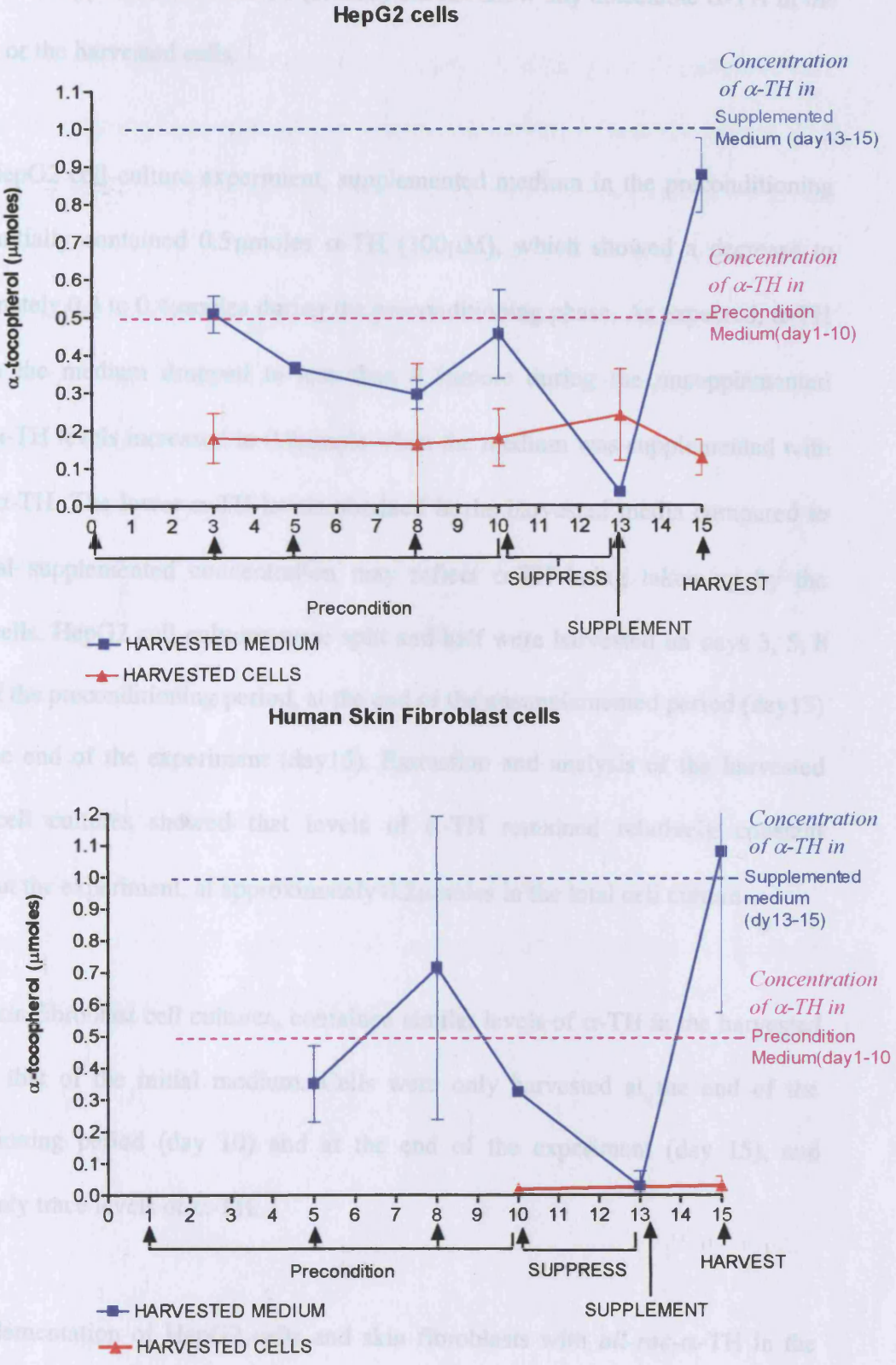


Figure 6.3; Amounts of all-rac-α-TH in cultured HepG2 cells and human skin fibroblasts and the respective spent medium, each point represents a mean of three flasks and error bars represent standard deviation. Arrow heads indicate feeding of

grown on unsupplemented medium (blanks) did not show any detectable α-TH in the medium or the harvested cells.

In the HepG2 cell culture experiment, supplemented medium in the preconditioning period initially contained 0.5μmoles α-TH (100μM), which showed a decrease to approximately 0.3 to 0.4μmoles during the preconditioning phase. As expected, α-TH levels in the medium dropped to less than 0.1μmole during the unsupplemented period. α-TH levels increased to 0.9μmole when the medium was supplemented with 200μM α-TH. The lower α-TH levels obtained in the harvested media compared to the actual supplemented concentration may reflect α-TH being taken up by the HepG2 cells. HepG2 cell cultures were split and half were harvested on days 3, 5, 8 and 10 of the preconditioning period, at the end of the unsupplemented period (day13) and at the end of the experiment (day15). Extraction and analysis of the harvested HepG2 cell cultures showed that levels of α-TH remained relatively constant throughout the experiment, at approximately 0.2μmoles in the total cell culture.

Human skin fibroblast cell cultures, contained similar levels of α-TH in the harvested media to that of the initial medium. Cells were only harvested at the end of the preconditioning period (day 10) and at the end of the experiment (day 15), and showed only trace levels of α-TH.

The supplementation of HepG2 cells and skin fibroblasts with *all-rac*-α-TH in the present study, showed that HepG2 cells in contrast to skin fibroblasts were able to take up α-TH from the supplemented growth medium.

Measurement of α-TH metabolites in spent media

The concentrations of α-TH metabolites were analysed in the growth medium of both cell lines and expressed per total protein concentration of the cells in the culture. This allowed the results of the present study to be standardised and compared to the results obtained by Birringer et al. (2001). It was found that HepG2 cells were able to metabolise α-TH to its side-chain shortened products, including α-CEHC, α-CMBHC and α-CMHHC, and then secrete into the growth medium. The detection of these three chain-shortened metabolites is shown by the chromatogram (figure 6.4). The time-course analysis for all three chain-shortened metabolites during the preconditioning and supplementation of the HepG2 cells with supplemented *all-rac*-α-TH is shown in figure 6.5. No α-TH metabolites could be detected in the spent medium of human skin fibroblasts. The concentration of metabolites remain relatively constant throughout the preconditioning period, where at day 8 the mean concentrations of, in particular the CMHHC and CMBHC are increased which may be due to the fact that at this point cultures had been incubated on the same supplemented medium for 3 days without harvesting or splitting of cell cultures compared to two days at all other harvest points.

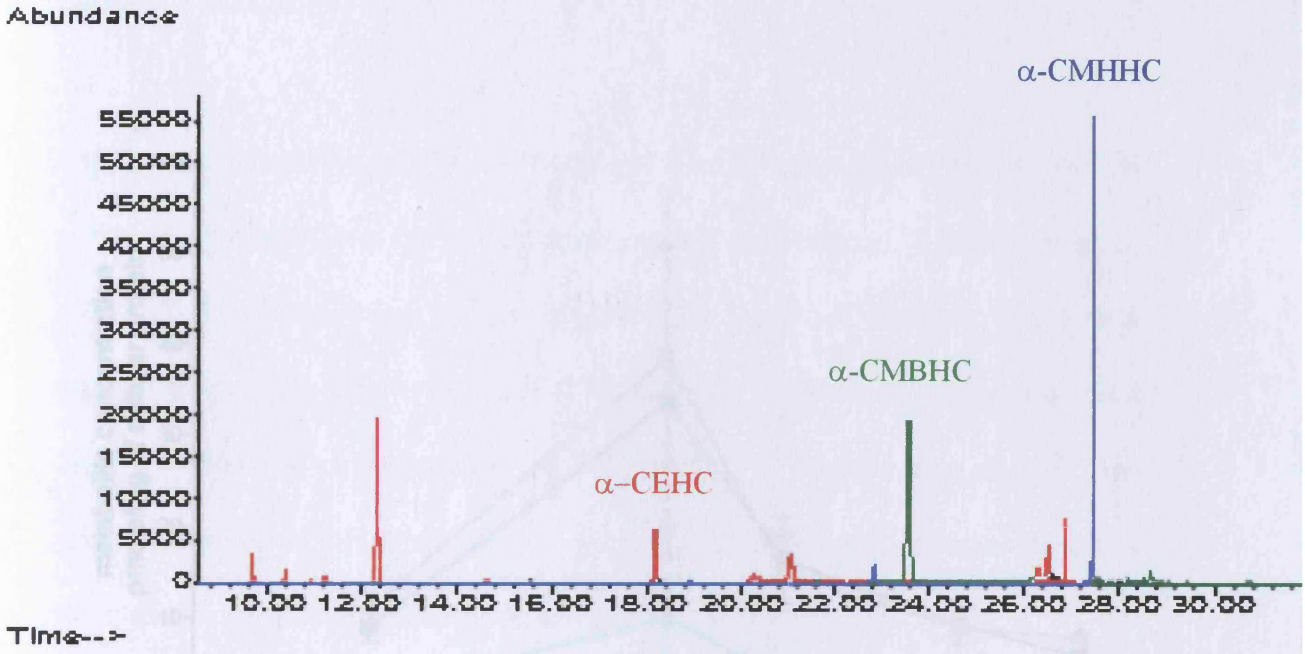


Figure 6.4; Chromatogram of extracted metabolites from HepG2 cell cultures fed all-rac-α-TH.

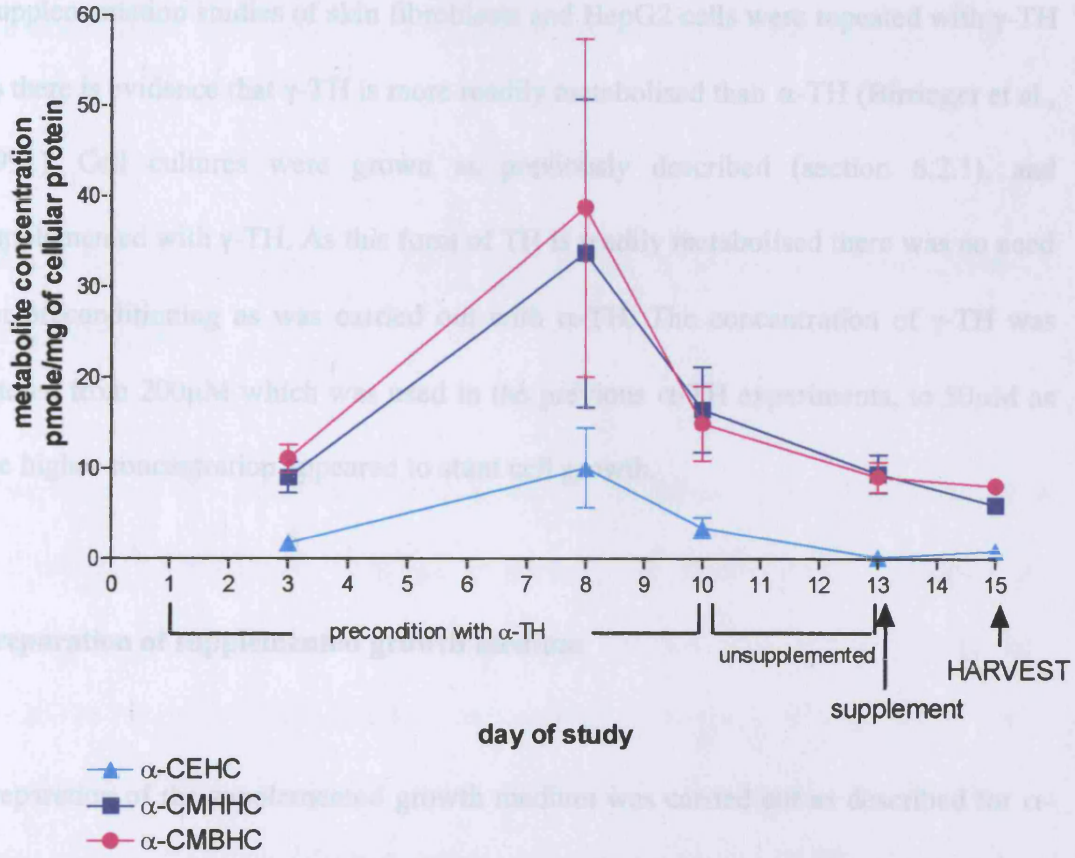


Figure 6.5; The concentrations of α-TH metabolites, α-CEHC, α-CMBHC and α-CMHHC, in the harvested media of HepG2 cell cultures fed with α-TH. Means and ranges of triplicate flasks are shown.

6.2.3 γ-Tocopherol supplementation of cell culture

6.2.3.1 γ-Tocopherol supplementation of human skin fibroblasts and HepG2 cells for 48 hours.

Supplementation studies of skin fibroblasts and HepG2 cells were repeated with γ-TH as there is evidence that γ-TH is more readily metabolised than α-TH (Birringer et al., 1991). Cell cultures were grown as previously described (section 6.2.1), and supplemented with γ-TH. As this form of TH is readily metabolised there was no need for preconditioning as was carried out with α-TH. The concentration of γ-TH was altered from 200μM which was used in the previous α-TH experiments, to 50μM as the higher concentration appeared to stunt cell growth.

Preparation of supplemented growth medium

Preparation of the supplemented growth medium was carried out as described for α-TH, in section 6.2.2.1 to give a final concentration of 50μM γ-TH in the growth medium. Concentrations of γ-TH supplemented into the FBS, and the final supplemented growth medium were measured using HPLC with fluorometric detection as described previously, before supplementing the cultures.

Feeding cultures supplemented medium

The study protocol was conducted, whereby three flasks of 85-90% confluent human skin fibroblast and HepG2 cell cultures were fed 50μM γ-TH supplemented medium for 48hrs. A fourth flask of each cell line was fed unsupplemented medium to provide a control culture. Cell cultures were harvested after 48 hours incubation.

Results following supplementation of human skin fibroblasts and HepG2 cells with γ-tocopherol

Concentrations of γ-TH in the cell suspension are as follows and are quoted in μM as cell protein measurements were not available. γ-TH concentrations in HepG2 cell suspensions had a mean value of 7.367 (n=3, SDEV=1.457) and for human skin fibroblast cell suspensions had a mean value of 9.335 (n=3, SDEV=3.508). These concentrations showed evidence of uptake of γ-TH by both cell lines. Metabolites were, however, only detected in the medium of the HepG2 cell cultures (figure 6.6). It can, therefore, be concluded that human skin fibroblasts are unable to metabolise the THs and/or secrete the resulting metabolites to a level that can be detected by the analytical method used. In the HepG2 cell culture medium, the three chain-shortened metabolites of γ–TH (γ-CEHC, γ-CMBHC and γ-CMHHC) were detected at similar concentrations (with means of 4.50, 6.25 and 4.20pmol/mg cell protein, respectively).

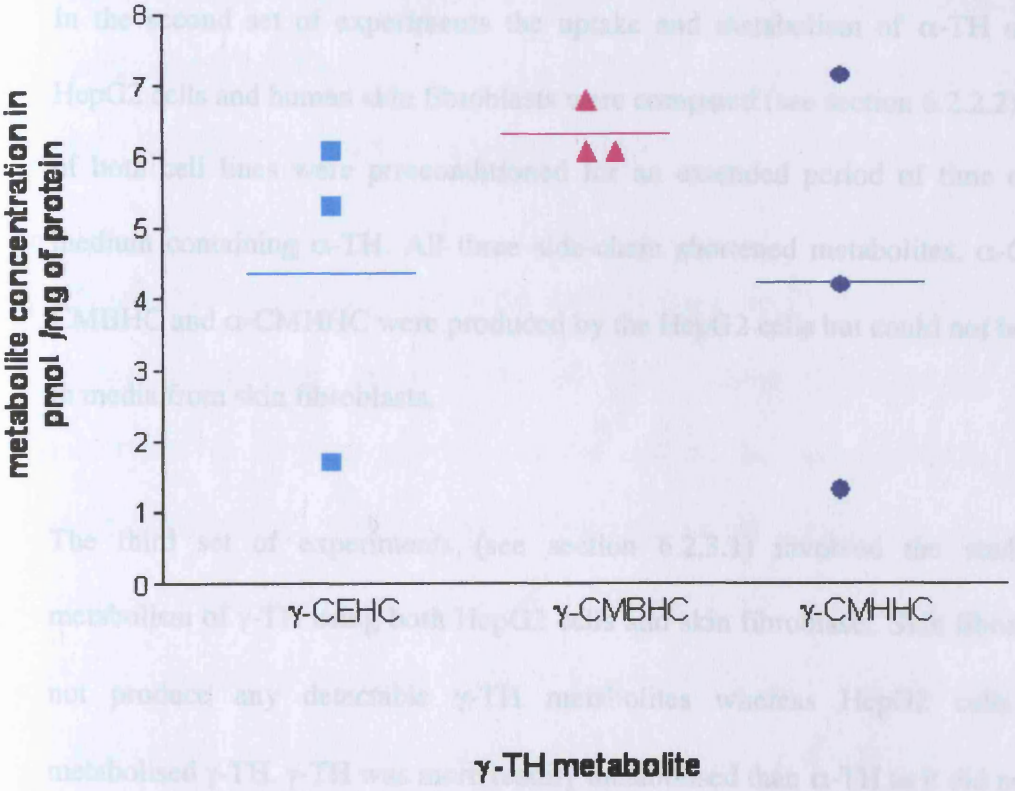


Figure 6.6; The concentrations of the γ-TH metabolites, γ-CEHC, γ-CMBHC and γ-CMHHC, in the harvested media of triplicate flasks of HepG2 cell cultures fed with 50μM γ-TH supplemented medium for 48 hours (means are indicated by the lines).

Discussion of results

In the preliminary experiments, healthy human control skin fibroblasts showed a very low uptake of α-TH into cells from the supplemented growth medium, and did not show any evidence of α-TH metabolism (see section 6.2.2.1).

In the second set of experiments the uptake and metabolism of α-TH using both HepG2 cells and human skin fibroblasts were compared (see section 6.2.2.2). Cultures of both cell lines were prreconditioned for an extended period of time on growth medium containing α-TH. All three side-chain shortened metabolites, α-CEHC, α-CMBHC and α-CMHHC were produced by the HepG2 cells but could not be detected in media from skin fibroblasts.

The third set of experiments (see section 6.2.3.1) involved the study of the metabolism of γ-TH using both HepG2 cells and skin fibroblasts. Skin fibroblasts did not produce any detectable γ-TH metabolites whereas HepG2 cells actively metabolised γ-TH. γ-TH was more readily metabolised than α-TH as it did not require the preconditioning stage necessary for the formation of detectable concentrations of α-TH metabolites. Supplementation with 50μM γ-TH for only 48hrs with no preconditioning yielded similar concentrations of the equivalent metabolites CEHC, CMBHC and CMHHC in the spent medium compared with 200μM α-TH and "preconditioning".

It has been previously reported in the literature that α-TH was not metabolised by HepG2 cells, although γ-TH was readily metabolised (Parker and Swanson, 2000). The findings of Parker and Swanson (2000) were confirmed by Birringer et al (2001) who re-examined α-TH metabolism by cultured HepG2 cells. They reported that the presence of γ-TH and δ-TH in the growth medium resulted in γ/δ-CEHC and CMBHC metabolites as secretory products in the spent medium, whereas α- and β-TH did not appear to be metabolised. Birringer's group further observed that preconditioning of the cultured cells with *all-rac*-α-TH for 10 days was necessary before any metabolites of α-TH could be detected in the growth medium.

The concentration of α-CEHC in the spent medium peaked on day 8 of the 10-day preconditioning with 100μM α-TH with give a mean of 10pmol/mg of protein (equivalent to 26ng/mg protein). At the end of the study following 48 hours incubation α-CEHC concentration was approximately 1pmol/mg protein in the spent medium. These values were similar to the α-CEHC concentrations of 20ng/mg protein reported by Birringer (2001) in studies involving a 10-day incubation with 100μM α-TH. In our study the longer chain metabolites, α-CMBHC and α-CMHHC, were present in higher concentrations with a mean of 39 and 32pmol/mg protein, respectively.

It was interpreted that the difference in handling of the different THs (α- and γ-TH in our study and α-, β-, δ- and γ-TH in Birringer's study) may result from either differences in the uptake of the THs by the HepG2 cells or differences in their metabolism. An adequate intracellular concentration of TH is obviously required

before metabolism can take place and as discussed previously (section 1.5.2) it is well recognised that α-TTP is highly selective for α-TH. This results in α-TH being specifically selected for transfer back into the circulation, whereas the other THs tend to be metabolised and excreted.

The relatively higher concentrations of the longer-chain metabolites, α-CMBHC and α-CMHHC may have been due to incomplete oxidation to α-CEHC, as a result of more rapid flux of the steps prior to the conversion step to α-CEHC. This metabolism of α-TH appeared to be different from γ-TH, where the concentrations of γ-CEHC, γ-CMBHC and γ-CMHHC were similar. The unsupplemented stage resulted in a decrease in metabolite levels detected in the spent medium, showing a rapid response in metabolism by the cells to α-TH availability in the growth medium.

The differences in the ability of HepG2 cells and human skin fibroblasts to handle α-TH may be explained by the fact that HepG2 cells are metabolically more active and specialised compared with skin fibroblasts. The failure of the skin fibroblasts to metabolise α-TH was therefore likely to be the result of inactivity of the relevant metabolic pathways and/or the low uptake of α-TH by this cell line.

The initial aim of the study was to investigate skin fibroblasts from patients, however, the inactivity of this cell line made it necessary to use other approaches. It was, therefore, decided to use HepG2 cells to investigate the subcellular organelle(s) involved in the metabolism of γ-TH.

6.2.4 Intracellular sites of tocopherol metabolism in HepG2 cells

HepG2 cells were incubated with γ-TH supplemented growth medium and both mitochondrial and peroxisomal activity were manipulated separately, in order to try and gain a better understanding of the localisation of the metabolism of the tocopherols.

As discussed in sections 6.1.1 and 6.1.2 it was decided to use etomoxir as a mitochondrial inhibitor, and PFOS as a peroxisomal proliferator.

6.2.4.1 γ-Tocopherol supplementation of HepG2 cells for 48 hours with manipulation of mitochondrial and peroxisomal function

Cell cultures were grown in four groups of three flasks and supplemented with γ-TH. One group was supplemented with γ-TH only and acted as a control. Addition of a mitochondrial inhibitor (20μM etomoxir) and/or a peroxisome proliferator (100μM PFOS) was made to the other three groups (figure 6.7).

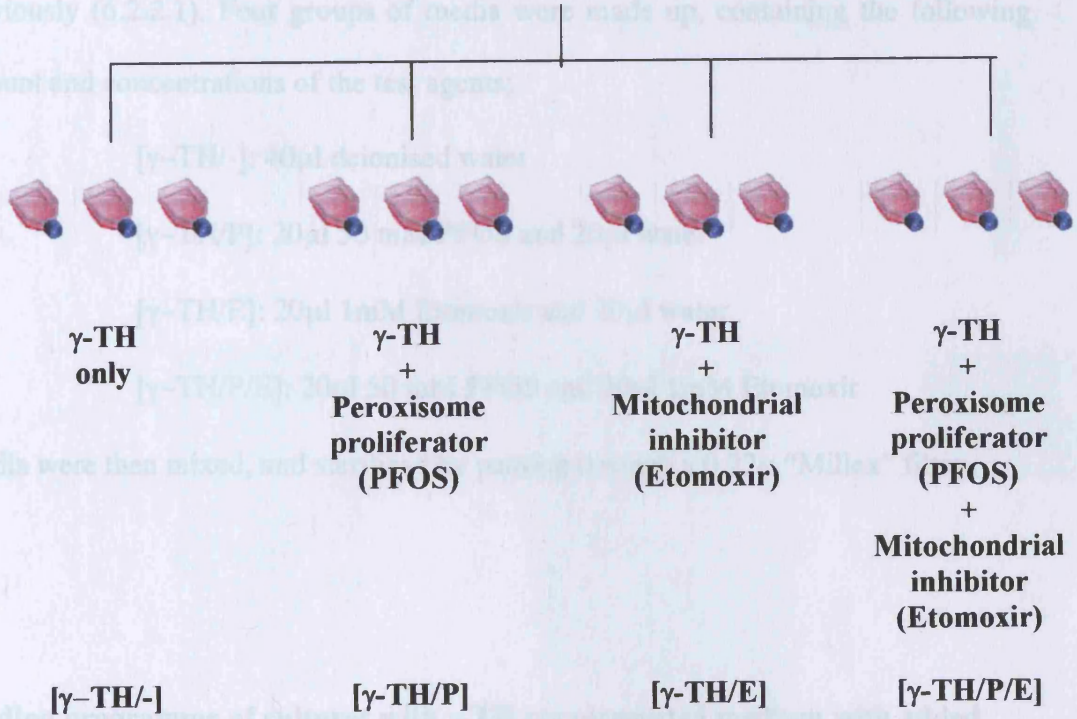


Figure 6.7; Supplementation of HepG2 cell cultures with γ-TH and mitochondrial inhibitor and/or peroxisome proliferator

Preparation of supplemented growth medium

Preparation of γ-TH supplemented growth medium was carried out as described previously (6.2.2.1). Four groups of media were made up, containing the following amount and concentrations of the test agents;

[γ–TH/-]: 40μl deionised water

[γ–TH/P]: 20μl 50 mM PFOS and 20μl water

[γ–TH/E]: 20μl 1mM Etomoxir and 20μl water

[γ–TH/P/E]: 20μl 50 mM PFOS and 20μl 1mM Etomoxir

Media were then mixed, and sterilized by passing through a 0.22μ "Millex" filter.

Feeding programme of cultures with γ-TH supplemented medium with added Etomoxir and/or PFOS

The feeding programme lasted for five days at the end of which culture media and cells were harvested (figure 6.8). The treatment of cell cultures during the feeding programme is described as follows.

Day 1- Twelve flasks of 85-90% confluent HepG2 cell cultures were grown on unsupplemented stock medium.

Three flasks where designated for each treatment group, [γ-TH/-], [γ-TH/P], [γ-TH/E] and [γ-TH/P/E]. 100μM PFOS was fed to the two appropriate cell culture groups, [γ-TH/P] and [γ-TH/P/E], to enable the action of this agent to be effective on the cell

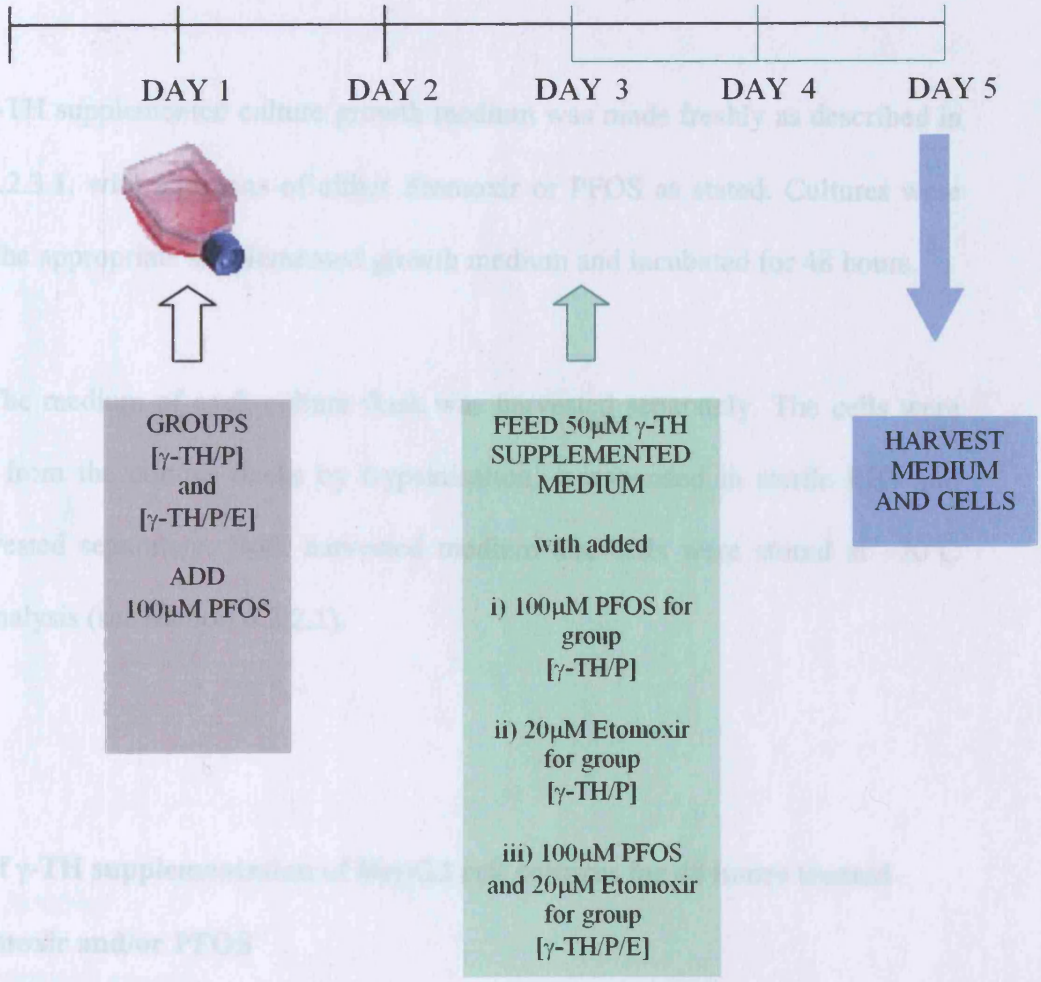


Figure 6.8; Feeding programme for HepG2 cell cultures with γ-TH supplemented medium with added etomoxir and/or PFOS

culture and allow time for proliferation of the peroxisomes of the cells before supplementing with γ-TH on day 3. All other cultures were fed with unsupplemented stock growth medium.

Day 3- γ-TH supplemented culture growth medium was made freshly as described in section 6.2.3.1, with additions of either Etomoxir or PFOS as stated. Cultures were fed with the appropriate supplemented growth medium and incubated for 48 hours.

Day 5- The medium of each culture flask was harvested separately. The cells were detached from the culture flasks by trypsinisation, resuspended in sterile PBS and then harvested separately. Both harvested medium and cells were stored at –20°C prior to analysis (see section 6.2.2.1).

Results of γ-TH supplementation of HepG2 cell cultures for 48 hours treated with Etomoxir and/or PFOS

All cultures were 95-100% confluent upon harvesting on day 5, and each culture flask contained a mean of 71mg cell protein.

Cellular uptake of γ-tocopherol by cell cultures

Figure 6.9 shows the concentration of γ-TH in the harvested cells and media from each individual flask in this study expressed as nmol per mg cell protein. The cellular concentrations in the control group gave similar concentrations in the triplicate flasks, in the range 1.28-1.45nmol/mg cell protein. The media concentrations of γ-TH were

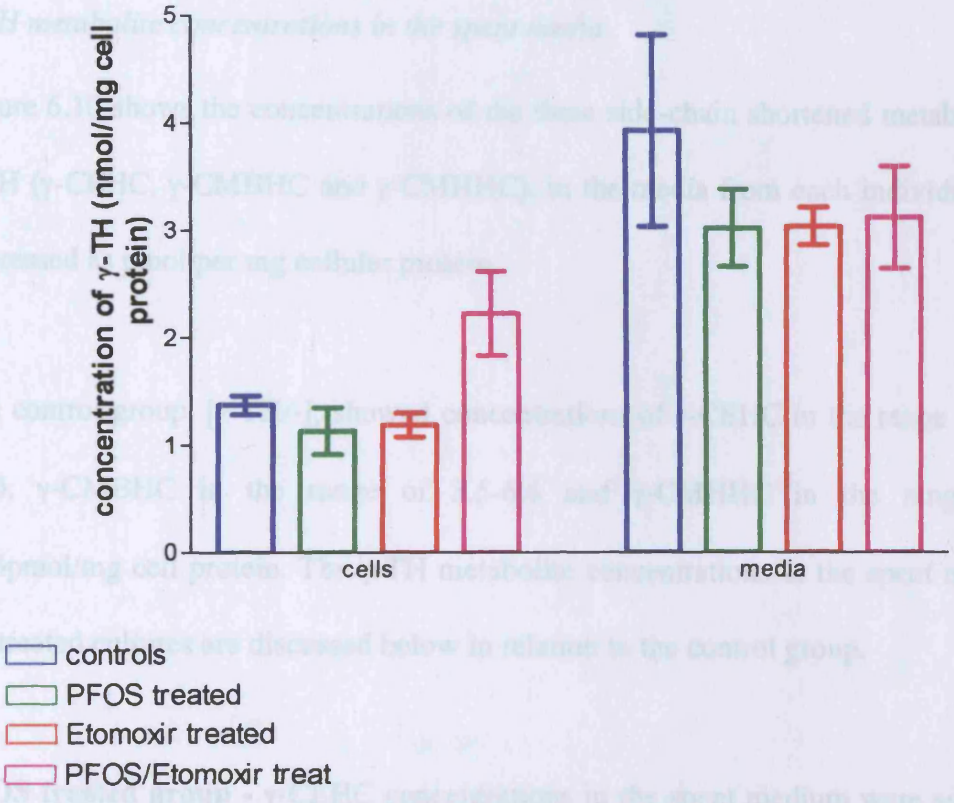


Figure 6.9; Concentrations of γ-TH in cells and media of HepG2 cell cultures fed with 50μM γ-TH supplemented media for 48hrs and treated with PFOS and/or etomoxir (each bar n=3).

only available for two of the three flasks and gave concentrations of 4.56 and 3.30nmol/mg cell protein. The γ-TH concentrations in the cells and media of the treated cultures showed similar concentrations to that of the control group except for the cellular concentration in the PFOS/etomoxir group which tended to be increased (1.78-2.48nmol/mg cell protein).

γ-TH metabolite concentrations in the spent media

Figure 6.10 shows the concentrations of the three side-chain shortened metabolites of γ-TH (γ-CEHC, γ-CMBHC and γ-CMHHC), in the media from each individual flask expressed as pmol per mg cellular protein.

The control group, [γ-TH/-], showed concentrations of γ-CEHC in the range of 11.4-17.3, γ-CMBHC in the range of 3.5-6.6 and γ-CMHHC in the range 1.77-3.13pmol/mg cell protein. The γ-TH metabolite concentrations in the spent media of the treated cultures are discussed below in relation to the control group.

PFOS treated group - γ-CEHC concentrations in the spent medium were similar to the control values in the range of 13.8-18.8pmol/mg cell protein. γ-CMBHC and γ-CMHHC concentrations appeared to be lower than that of the control group, in the ranges 0.6-1.8 and 0.3-0.6pmol/mg cell protein, respectively. The data set is very small and is therefore only insufficient to carry out limited statistical analyses. The overall affect of the different treatments on the three individual metabolites was statistically analysed using GLM univariate analysis with Bonferonni post test (SPSS software). These tests gave the following results; CEHC was not significantly affected

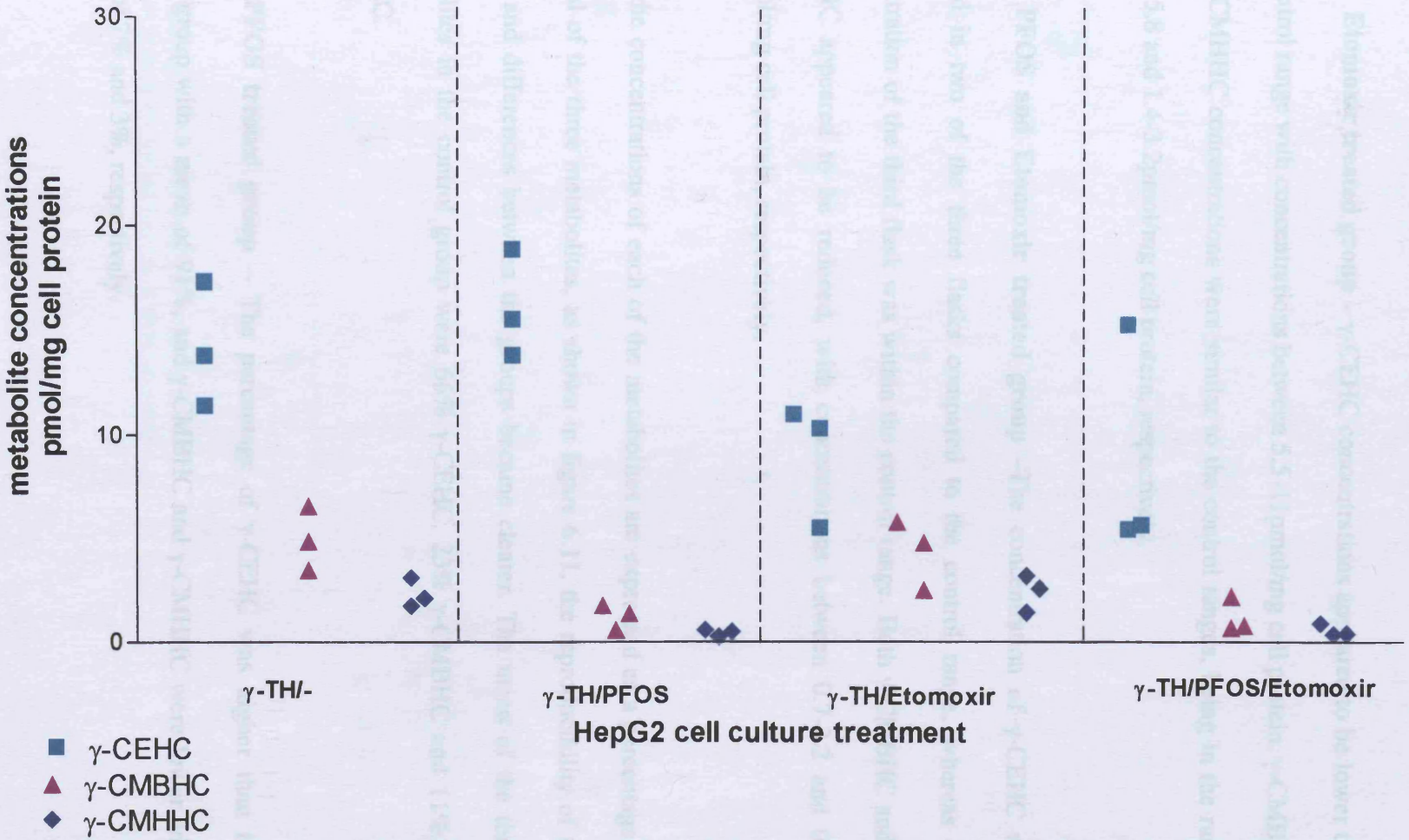


Figure 6.10; Concentrations of γ-TH metabolites, γ-CEHC, γ-CMBHC and γ-CMHHC, in the harvested media of the four groups. Each point represents one flask.

according to the Null hypothesis (p=0.104), CMBHC and CMHHC were significantly affected (p= 0.038 and p= 0.040 respectively).

Etomoxir treated group – γ-CEHC concentrations appeared to be lower than the control range with concentrations between 5.5-11pmol/mg cell protein. γ-CMBHC and γ-CMHHC concentrations were similar to the control ranges, being in the range of 2.5-5.8 and 1.4-3.2pmol/mg cell protein, respectively.

PFOS and Etomoxir treated group –The concentration of γ-CEHC was reduced in two of the three flasks compared to the control range, whereas the concentration of the third flask was within the control range. Both γ-CMBHC and γ-CMHHC appeared to be reduced, with concentrations between 0.7-2.2 and 0.4-0.9nmol/mg cell protein, respectively.

When the concentrations of each of the metabolites are expressed as a percentage of the total of the three metabolites, as shown in figure 6.11, the reproducibility of the results and differences between the groups became clearer. The mean of the three metabolites in the control group were 66% γ-CEHC, 23% γ-CMBHC and 11% γ-CMHHC.

PFOS treated group – The percentage of γ-CEHC was higher than the control group with a mean of 91%, and γ-CMBHC and γ-CMHHC were lower with means of 7% and 3%, respectively.

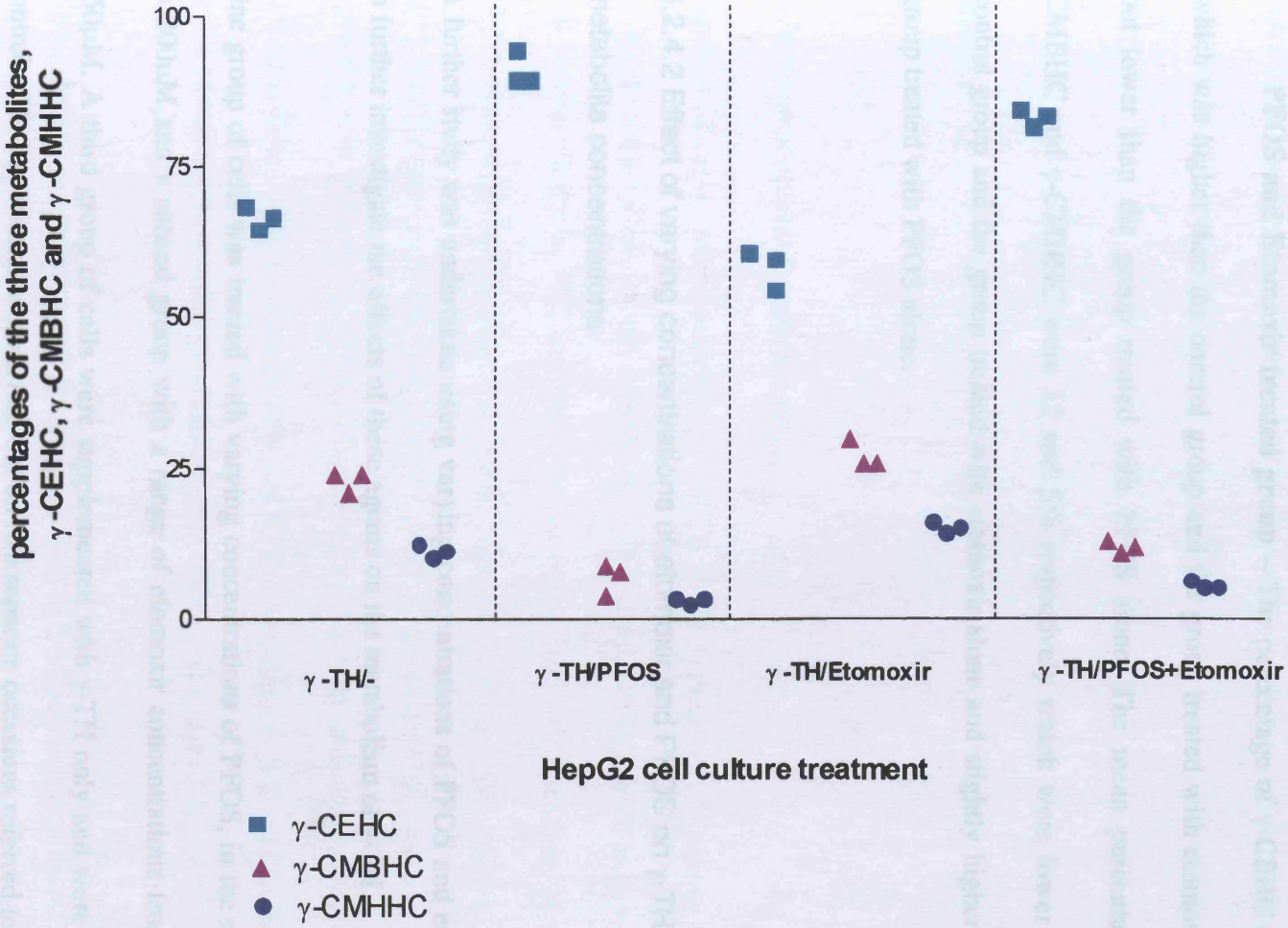


Figure 6.11; Percentages of the three chain shortened metabolites in the harvested media of the four groups. Each individual point represents one flask.

Etomoxir treated group – The percentage of γ-CEHC appeared to be lower than the control group with a mean of 58%, and γ-CMBHC and γ-CMHHC had means of 23% and 15% respectively.

PFOS and Etomoxir treated group – The percentage of γ-CEHC was 83% which was higher than the control group and the group treated with etomoxir alone, but lower than the group treated with PFOS alone. The mean percentage of γ-CMBHC and γ-CMHHC were 12 and 5% respectively which were lower than the control group and the group treated with etomoxir alone and slightly higher than the group treated with PFOS alone.

6.2.4.2 Effect of varying concentrations of etomoxir and PFOS on γ-TH metabolite concentrations

A further study was undertaken using varying concentrations of PFOS and etomoxir, to further investigate the effects of these agents on the metabolism of γ-TH.

One group of cells was treated with varying concentrations of PFOS, in the range 50 to 200μM and a second group with a range of etomoxir concentrations from 20 to 150μM. A third group of cells were supplemented with γ-TH only and were used as controls. The experiment was carried out on two separate occasions referred to as TC2 and TC3.

Experimental protocol

The feeding programme for the HepG2 cell cultures was performed as described in section 6.2.4.1. Eleven cell culture flasks at 85-90% confluency were selected, where three were used as control flasks, four were treated with PFOS and the remaining four were treated with etomoxir.

Results

γ-Tocopherol uptake by cells in culture

Figure 6.12 shows the concentration of γ-TH in the harvested cells and in the spent media from each flask in both experiments (TC2 and TC3) expressed as nmol per mg cell protein. Firstly in TC2, the control group had cellular concentrations, in the range 1.02-1.90nmol/mg cell protein. The media concentrations of γ-TH were in the range of 3.05-5.58nmol/mg cell protein. In TC3, the control group had cellular concentrations in the range 2.00-2.32nmol/mg cell protein and media concentrations of 4.62-4.93nmol/mg cell protein. The γ-TH concentrations in the cells and the spent media of the treated cultures gave similar concentrations to that of the control group.

Although the concentrations of the etomoxir and PFOS treated groups in TC3 tended to be greater than the controls, there were no consistent trends with increasing concentrations of the PFOS or etomoxir.

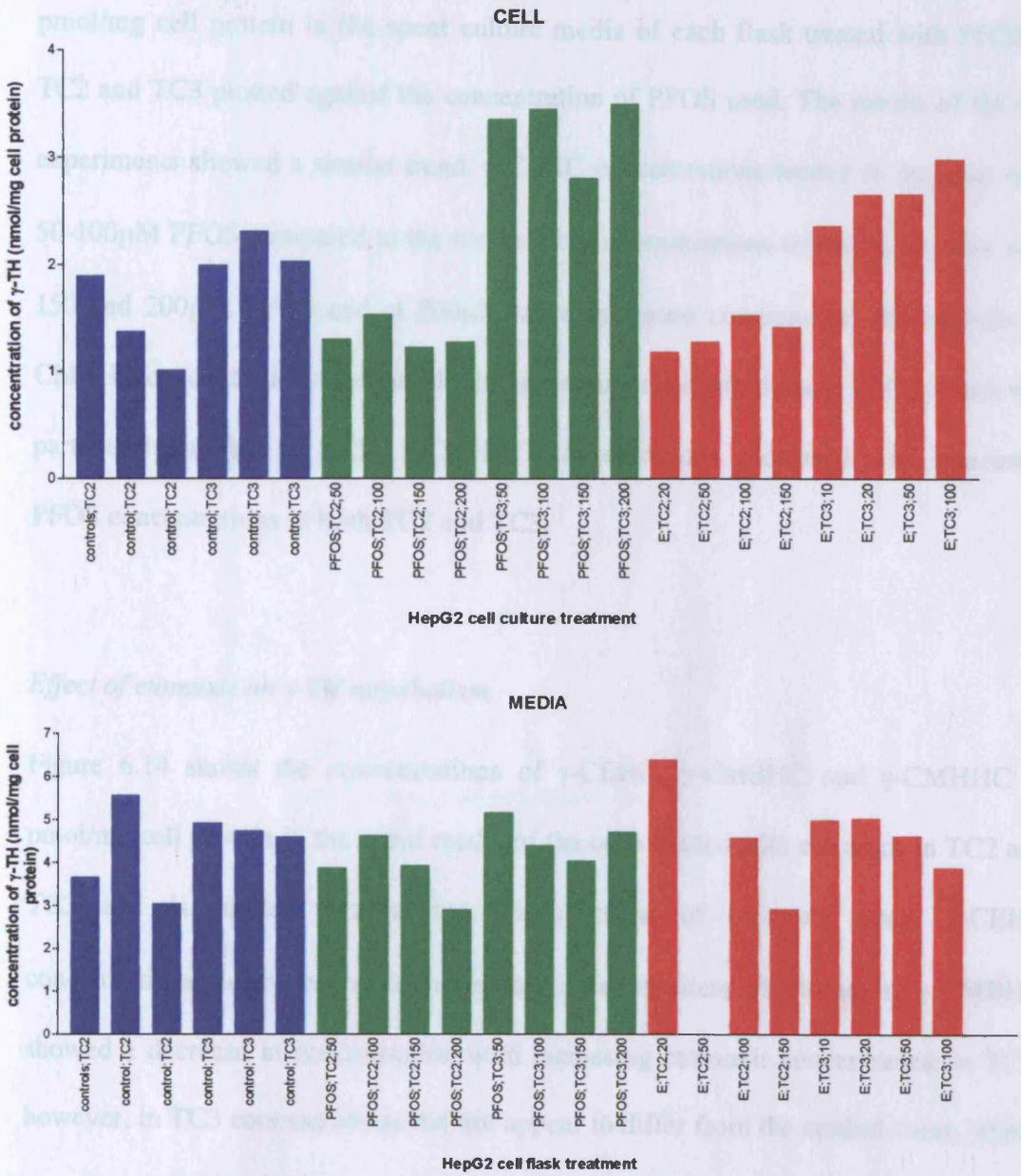


Figure 6.12; Concentrations of γ-TH in cells and media of HepG2 cell cultures fed with 50μM γ-TH and treated with varying concentrations of either PFOS or etomoxir (each bar n=1).

Effect of PFOS concentrations on γ-TH metabolites

Figure 6.13 shows the concentrations of γ-CEHC, γ-CMBHC and γ-CMHHC pmol/mg cell protein in the spent culture media of each flask treated with PFOS in TC2 and TC3 plotted against the concentration of PFOS used. The results of the two experiments showed a similar trend. γ-CEHC concentrations tended to decrease with 50-100μM PFOS compared to the controls but concentrations tended to increase with 150 and 200μM PFOS and at 200μM were increased compared to the controls. γ-CMBHC concentrations decreased with increasing concentrations of PFOS which was particularly marked in TC2. γ-CMHHC concentrations decreased with increasing PFOS concentrations in both TC2 and TC3.

Effect of etomoxir on γ-TH metabolism

Figure 6.14 shows the concentrations of γ-CEHC, γ-CMBHC and γ-CMHHC in pmol/mg cell protein in the spent media of the cells treated with etomoxir in TC2 and TC3 and is plotted against the concentration of etomoxir used. γ-CEHC concentrations decreased with increasing concentrations of etomoxir. γ-CMBHC showed a decrease in concentration with increasing etomoxir concentration in TC2, however, in TC3 concentrations did not appear to differ from the control value, which was also much lower than the control in TC2. Similarly, γ-CMHHC concentrations showed a decrease in response to increasing etomoxir concentrations in TC2, but no significant changes in TC3.

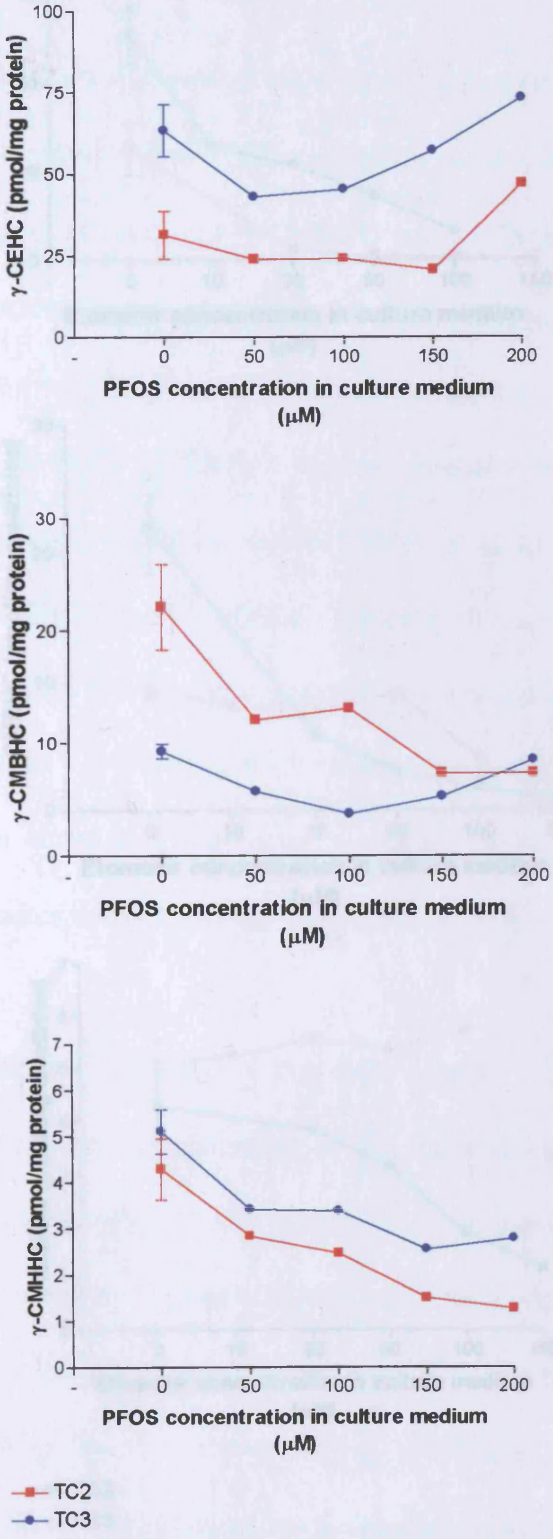


Figure 6.13; The effect of increasing concentrations of PFOS on the concentration of each of the three γ-TH chain shortened metabolites in TC2 and TC3. Means and SD are shown for the control cells (n=3).

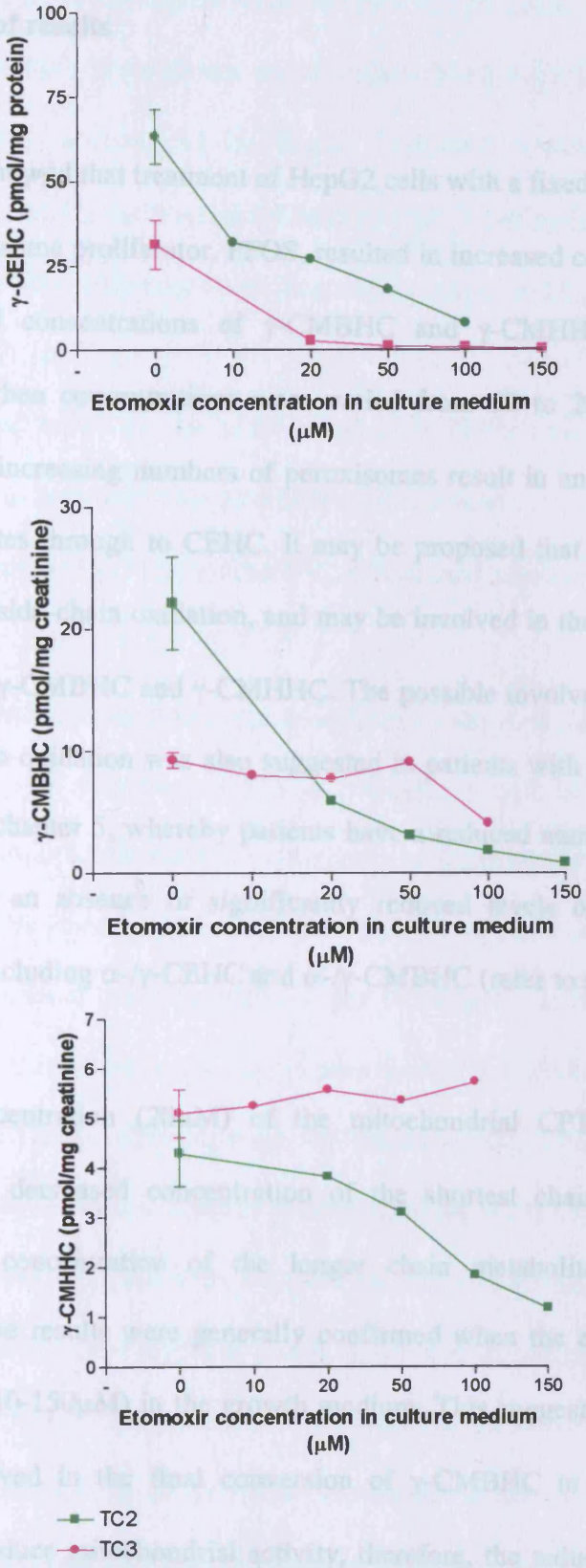


Figure 6.14; The effect of increasing concentrations of etomoxir on the concentration of each of the three γ-TH chain shortened metabolites in TC2 and TC3. Means and SD are shown for the control cells (n=3).

Discussion of results

This study showed that treatment of HepG2 cells with a fixed concentration (100 μM) of the peroxisome proliferator, PFOS, resulted in increased concentrations of γ-CEHC and reduced concentrations of γ-CMBHC and γ-CMHHC. This was generally confirmed when concentrations were varied from 50 to 200μM PFOS. This may suggest that increasing numbers of peroxisomes result in an increased flux of the γ-TH metabolites through to CEHC. It may be proposed that the peroxisomes have a role in γ-TH side-chain oxidation, and may be involved in the conversion steps in the formation of γ-CMBHC and γ-CMHHC. The possible involvement of peroxisomes in TH side-chain oxidation was also suggested in patients with Zellweger Syndrome as discussed in chapter 5, whereby patients have a reduced number of peroxisomes and demonstrated an absence or significantly reduced levels of urinary α- and γ-TH metabolites including α-/γ-CEHC and α-/γ-CMBHC (refer to section 5.2.3.2).

A fixed concentration (20μM) of the mitochondrial CPT-I inhibitor, etomoxir, resulted in a decreased concentration of the shortest chain metabolite, γ-CEHC, whereas the concentration of the longer chain metabolites remained relatively constant. These results were generally confirmed when the etomoxir concentrations were varied (10-150μM) in the growth medium. This suggests that the mitochondria may be involved in the final conversion of γ-CMBHC to γ-CEHC. Etomoxir is expected to reduce mitochondrial activity, therefore, the reduced concentration of γ-CEHC and unchanged concentration of the longer chain metabolites γ-CMBHC and γ-CMHHC may suggest mitochondrial involvement in the conversion of γ-CMBHC to

γ-CEHC. This would agree with studies of pristanic acid metabolism where mitochondria have been shown to be responsible for the final cycles of side-chain oxidation (see section 5.1.4). Higher etomoxir concentrations of 50-100μM corresponded with a decrease in γ-CMBHC and γ-CMHHC, which may be explained by a possible toxic effect, however, this only occurred in TC2.

The combined effect of the addition of both PFOS and etomoxir appears to be intermediate to that exhibited by either of the treatments alone, whereby γ-CEHC is elevated and γ-CMBHC and γ–CMHHC are reduced relative to the controls.

When interpreting these results the specificity of the modulators must be considered. Although in the literature, PFOS is thought to act as a specific peroxisome proliferator, etomoxir is known to increase the expression of the gene encoding the rate-limiting enzyme, acyl-CoA oxidase (ACO), of peroxisomal β-oxidation. Thus studies have shown that coupled with the decrease in mitochondrial FA oxidation there is also an observable increase in peroxisomal FA oxidation upon the use of the mitochondrial CPT-I inhibitor, etomoxir (Skorin et al., 1992). It is thought that the intracellular accumulation of lipids following inhibition of CPT-I activity by etomoxir leads to a peroxisome proliferator associated receptor, PPAR-α, mediated increase in the expression of genes involved in alternative FA oxidation pathways. An example of this is detection of an increase in mRNA expression of acyl-CoA oxidase (ACO), a rate limiting enzyme of peroxisomal β-oxidation (Cabrero et al., 1999). Thus the results obtained using such agents should be interpreted with some caution.

This chapter has dealt with the metabolism of γ-TH, by human HepG2 cells. Having established that three successive chain shortened metabolites, CMHHC, CMBHC and CEHC are produced and excreted in to the culture medium, the manipulation of the mitochondria and peroxisomes showed that both these organelles play a role in γ-TH metabolism. Proliferation of the peroxisomes using the peroxisome proliferator, PFOS, resulted in an increase in γ-CEHC which supports the results obtained for the Zellweger patient's urine studied in chapter 5. Inhibition of the import of the shortened chain metabolites CMBHC and/ or CMHHC into the mitochondria using a CPT-I inhibitor, Etomoxir, resulted in a decrease in γ-CEHC. It therefore appears that γ-TH is initially metabolized by the peroxisome and the later stages of metabolism leading to the shortest chain metabolite CEHC is carried out by the mitochondrion.

Chapter 7

Conclusion and future work

The aims of my study were to investigate 1) whether α-TL can be used as a potential biomarker of in vivo oxidative stress and 2) the cellular localisation of the side-chain shortening processes involved in TH conversion to the urinary metabolic products (CEHC, CMBHC and CMHHC).

7.1 α-Tocopheronolactone – a potential biomarker of oxidative stress?

α-Tocopheronolactone (α-TL) was first discovered by Simon et al (1956) and initially thought to be one of the major metabolites of TH in human urine. Subsequent studies have found that chain shortened metabolites with an unoxidised ring, CEHC and CMBHC are the major urinary metabolites (Chiku et al., 1984) and that α-TL along with the other 'Simon metabolites" may be products of artefactual oxidation during the analytical procedure. The addition of the internal standard, deuterated α-CEHC, prior to sample preparation enabled the estimation of artefactual oxidation as shown by the conversion of the deuterated α-CEHC standard to deuterated α-TL. Our studies also demonstrated that there was a consistently higher concentration of undeuterated α-TL than that calculated as a product of artefactual conversion by deuterated α-CEHC standard to α-TL. This difference may represent endogenous α-TL and, therefore, may offer a potential biomarker of in vivo oxidative stress.

Methods were established to measure and compare urinary α-TL and 8-isoprostane (an independent biomarker of in vivo oxidative stress) from individuals taking part in a strenuous exercise regimen. Although good correlations between α-TL and 8-isoprostane were demonstrated, the groups chosen for this study, unfortunately, did not show any evidence of oxidative stress over a period of. A response could have been missed as the study was continued for 48 hours following exercise and Sacheck et al (2003) have reported that plasma F_2-isoprostane levels were only elevated after 72 hours post exercise. In retrospect exercise is a complex process and involves a number of mechanisms including adaptation by upregulation of antioxidant defences and repair systems (Radak et al., 2002; Svensson et al., 2002).

Possible future work might involve a similar urinary analysis of other groups of individuals, such as sepsis patients, who have been consistently shown to have increased in vivo oxidative stress (Takeda et al., 1984; Goode et al., 1995; Galley et al., 1996). An alternative methodology may also be used, whereby, the conjugates of vitamin E metabolites are directly analysed by tandem mass spectrometry (MS-MS). MS-MS enables the analysis of metabolites without their deconjugation and derivatisation to trimethylsilyl derivatives which is required for GC-MS methods. Using MS-MS, endogenous urinary conjugates of α-CEHC cannot undergo artefactual oxidation to form α-TL. Any α-TL detected must, therefore, be a real product formed in vivo from α-TH and excreted in urine. If α-TL is established as an authentic metabolite and can be measured accurately, it may be useful as a non-invasive marker of oxidative stress and used to monitor disease progression and the effects of interventions such as nutritional supplementation or drugs.

7.2 Localisation of tocopherol metabolism

The detection of vitamin E metabolites has provided an insight into pathways of vitamin E metabolism. The principal metabolites in human urine and plasma are the chroman ring intact and side-chain oxidised metabolites including δ-, γ-, and α-CEHC (Chiku et al., 1984; Schultz et al., 1995; Wechter et al., 1996). It was postulated that THs undergo sequential side-chain shortening reactions prior to their removal from the body. Sequential side-chain shortening by β-oxidation to produce CEHC is supported by the presence of precursors, such as CMBHC and CMHHC in human urine and in spent culture medium of HepG2 cells grown on γ-TH (Sontag and Parker, 2002; Pope et al., 2002; Birringer et al., 2002).

The sub-cellular localisation of the metabolism of TH has not yet been fully characterised. The initial ω-oxidation is a prerequisite for β-oxidation, whereby the terminal methyl group of the vitamin E side-chain is oxidised to provide a suitable substrate for β-oxidation. Studies using inhibitors of various cytochrome P450 (CYP) enzymes have indicated that this ω-oxidation of α- and γ-TH is CYP 3A dependent and occurs in the microsomes (Parker et al., 2000; Birringer et al., 2001;Ikeda et al., 2002). The cellular pathway and location of β-oxidation of the vitamin E side-chain have not been fully characterised, but it has been postulated that it occurs in the peroxisomes or mitochondria or in both organelles (as is the case for phytanic acid).

Originally two approaches were used to investigate the cellular localisation of tocopherol metabolism in vivo. The first of these approaches involved the use of patient material in the form of both skin fibroblasts derived from skin biopsy and urine. It was concluded that skin fibroblast studies were not possible due to the apparent lack of TH metabolism by this cell line, which may be due to its inherent metabolic inactivity. Alternatively, inadequate amount of the free phenolic form of tocopherol, which was supplemented to the growth medium, may have been taken up by the fibroblasts and transported to the sites of metabolism to enable side-chain shortening to proceed. Future work may include the use of acetate esters of TH i.e. α-tocopheryl acetate (α-TAc) which is commonly used in oral vitamin E supplements. α-TAc is more stable than α-TH and has been shown to be better absorbed by the skin than the free phenolic form of TH (Beijersbergen van Henegouwen et al., 1995; Mavon et al., 2004). Although supplementing fibroblasts with α-TAc may result in an improved uptake, the capability of this cell line to metabolise the side chain of the tocopherols remains unknown.

The analysis of urinary vitamin E metabolites from patients with mitochondrial and peroxisomal disorders were inconclusive. Although two patients diagnosed with Zellweger Syndrome (with a greatly reduced number of peroxisomes), showed an absence of α- and γ-TH urinary metabolites, concentrations of β- and δ-TH metabolites were within normal control ranges. This suggested a possible role of the peroxisomes in the metabolism of α- and γ-TH, and that β- and δ-TH may be metabolised by different pathways. The only other patient that demonstrated a metabolite profile that deviated from the control ranges was a patient diagnosed with Pearson's-like syndrome, with a defective mitochondrial respiratory chain. There was

an absence of α-TH metabolites in the urine, whereas the other metabolites were within normal ranges, suggesting the possible involvement of the mitochondria in the metabolism of α-TH. The inconclusive nature of these results suggested there was sufficient residual activity of the mitochondrial and peroxisomal systems in these patients, and that a total absence of activity of either systems may be incompatible with life.

An alternative approach to the study of the localisation of TH metabolism was the use of HepG2 cells and modulators of mitochondrial and peroxisomal activity. HepG2 cells grown on γ-TH supplemented growth medium and treated with a mitochondrial inhibitor (etomoxir) and/or a peroxisome proliferator (PFOS) gave some indication of the involvement of these organelles in γ-TH metabolism. The inhibition of the mitochondrial CPT-I activity by etomoxir resulted in the reduced formation of the end product γ-CEHC and some accumulation of γ-CMBHC and γ-CMHHC, suggesting involvement in the formation of γ-CEHC. This was coupled, however, with a decrease in the total concentration of the metabolites detected, a possible result of reduced activity of overall metabolism in the cells due to reduced mitochondrial activity. Peroxisome proliferation of this cell line appeared to cause an increase in the metabolic flux of γ-TH demonstrated by a decrease in the longer chain length metabolites (CMBHC and CMHHC) and an increase in the shortest chain metabolite (CEHC) suggesting a role in the formation of γ-CEHC via γ-CMBHC and γ-CMHHC.

Future work to investigate the subcellular localisation of TH metabolism may include mitochondrial or peroxisomal enzyme knock-out studies. In addition further α-/γ-TH supplementation studies with HepG2 cells may be carried out to investigate whether

there are any significant changes in the expression of proteins in mitochondrial and/or peroxisomal organelle fractions. Such studies may lead to the characterisation of the enzymes and other proteins involved in the cellular uptake and metabolism of the THs.

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